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<p>(21) International Application Number: <b>PCT/DK98/00076</b></p> <p>(22) International Filing Date: 26 February 1998 (26.02.98)</p> <p>(30) Priority Data:              0212/97                      26 February 1997 (26.02.97)      DK              60/051,016                27 May 1997 (27.05.97)              US</p> <p>(71) Applicant (for all designated States except US): <b>NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK).</b></p> <p>(72) Inventor; and</p> <p>(75) Inventor/Applicant (for US only): <b>NIELSEN, Ruby, Illum [DK/DK]; Gedebakken 9, DK-3520 Farum (DK).</b></p> <p>(74) Common Representative: <b>NOVO NORDISK A/S; Corporate Patents, Novo Allé, DK-2880 Bagsværd (DK).</b></p>		<p>(81) Designated States: <b>AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</b></p> <p><b>Published</b>  <i>With international search report.</i></p>
<p>(54) Title: <b>MICROBIAL XYLOGLUCAN ENDOTRANSGLYCOSYLASE (XET)</b></p> <p>(57) Abstract</p> <p>It has been found by a screening assay that XET activity is produced by an overwhelming array of phylogenetically dispersed microorganisms. Accordingly, the present invention relates to a xyloglucan endotransglycosylase preparation which is producible by cultivation of a microorganism expressing an XET.</p>		

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5   **MICROBIAL XYLOGLUCAN ENDOTRANSGLYCOSYLASE (XET)**

**FIELD OF THE INVENTION**

The present invention relates to microbial xyloglucan endotransglycosylases (XETs) and the production and uses thereof.

10   **BACKGROUND OF THE INVENTION**

Xyloglucan endotransglycosylase (XET) is an enzyme known from plants. To our best knowledge XET has never been described from microorganisms.

15   Stephen C. Fry et al. suggest in Biochem. J (1992) 282, p. 821-828 that XET is responsible for cutting and rejoining intermicrofibrillar xyloglucan chains and that XET thus causes the wall-loosening required for plant cell expansion.

XET has been suggested for use in regulating the morphology of a plant, see EP 562 836 p. 2 l. 27-28.

20   XET is believed to be present in all plants, in particular in all land plants. XET has been extracted from dicotyledons, monocotyledons, in particular graminaceous monocotyledons and liliaceous monocotyledons, and also from a moss and a liverwort, for reference see Biochem. J (1992) 282, p. 823.

30   In the copending patent application PCT/DK 96/00538 (WO 97/23683) we have shown that a cellulosic material may get improved strength properties and/or improved shape-retention properties and/or improved anti-wrinkling properties after treatment with an XET enzyme.

35   The XET enzyme has some very interesting applications, so it is an object of the present invention to provide microbially derived xyloglucan endotransglycosylases useful for, e.g., the applications described above. The microbially derived xyloglucan endotransglycosylases would have the great advantage that they could easily be produced in great quantities.

**BRIEF DISCLOSURE OF THE INVENTION**

5           It has been found by a screening assay that XET activity is produced by an overwhelming array of phylogenetically dispersed microorganisms.

          Accordingly, the present invention relates to a xyloglucan  
10 endotransglycosylase preparation which is producible by cultivation of a microorganism expressing an XET; in particular the present invention relates to:

A method for the production of a xyloglucan endotransglycosylase  
15 enzyme (XET) comprising

(a) culturing in a suitable nutrient medium a microorganism expressing a microbial XET under conditions conducive to the production of the XET enzyme, and

(b) subsequently recovering of the XET enzyme from the nutrient  
20 medium.

          Further, the present invention relates to use of the XET preparation of the present invention for treating cellulosic material, and to microbial XET preparations as such.

#### 25           BRIEF DESCRIPTION OF DRAWINGS

          The present invention is further illustrated by reference to the accompanying drawing, in which

30           Fig. 1 shows the pH profiles of *Dichotomocladium hesseltinei*, *Tiarosporella phaseolina* and *Pseudoplectania nigrella* XET activity ( $\Delta$ :*Tiarosporella phaseolina*;  $\square$ :*Pseudoplectania nigrella*; and  $\blacklozenge$ :*Dichotomocladium hesseltinei*) obtained according to Example 5.

35           Fig. 2 shows the pH profiles of *Dichotomocladium hesseltinei*, *Tiarosporella phaseolina* and *Pseudoplectania nigrella* xyloglucanase activity ( $\Delta$ :*Tiarosporella phaseolina*;  $\square$ :*Pseudoplectania nigrella*; and  $\blacklozenge$ :*Dichotomocladium hesseltinei*) obtained according to Example 5.

5

**DETAILED DISCLOSURE OF THE INVENTION**

The present invention relates to a xyloglucan endotransglycosylase preparation which is producible by cultivation of a microorganism.

It has been shown in the present application that XET is produced by fungi and bacteria, and it is contemplated that XET is also produced by yeasts.

An "XET-paper" was used for identifying XET producing microorganisms. The "XET-paper" is described in the copending patent application PCT/GB96/02351 (WO 97/11193); it is a xyloglucan-coated paper which is dipped through a labelled oligosaccharide.

The labelled oligosaccharide, the xyloglucan-impregnated paper, and the dot-blot test for XET activity were made in the following way:

Preparation of labelled oligosaccharide

The reducing oligosaccharide 4-O-[4-O-[4-O-[6-O- $\alpha$ -D-Xylopyranosyl- $\beta$ -D-glucopyranosyl]-6-O-(2-O- $\beta$ -D-galactopyranosyl)- $\alpha$ -D-xylopyranosyl- $\beta$ -D-glucopyranosyl]-6-O-(2-O- $\beta$ -D-galactopyranosyl)- $\alpha$ -D-xylopyranosyl- $\beta$ -D-glucopyranosyl]-D-glucose ("XLLG") (1 gram) is dissolved in 25 ml of a saturated aqueous solution of ammonium hydrogencarbonate containing 1 gram of sodium cyanoborohydride (NaCNBH<sub>3</sub>) and incubated in the dark at 25°C for 7 days to permit reductive amination. The ammonium hydrogencarbonate is then removed by drying, and the (ninhydrin-reactive) aminated derivative of XLLG is purified e.g. by gel-permeation chromatography or cation-exchange chromatography. The product is believed to be an oligosaccharidyl-1-amino-1-deoxyalditol, i.e. a derivative of XLLG in which the reducing terminal D-glucose moiety has been replaced by 1-amino-1-deoxy-D-glucitol.

5       The oligosaccharidyl-1-amino-1-deoxyalditol (50mg) is  
dissolved in 3 ml of 3% borax (di-sodium tetraborate; pH = 9.0-  
9.5) and a freshly-prepared solution of 10 mg lissamine rhodamine  
sulphonyl chloride [purchased from Molecular Probes Inc., USA] in  
10 0.75 ml of dry dimethylformamide (DMF) is added gradually, with  
stirring, and the mixture is incubated in the dark overnight. A  
further 0.75 ml of DMF containing 10 mg lissamine rhodamine  
sulphonyl chloride is added and the mixture incubated for a  
further 8 h. The bright pink oligosaccharidyl-1-amino-1-  
deoxyalditol-lissamine-rhodamine conjugate (XLLGol-SR) is  
15 purified by gel-permeation chromatography followed by reversed-  
phase chromatography on a C<sub>18</sub>-silica gel column. After washing of  
the latter column with water, a methanol gradient is applied and  
the XLLGol-SR elutes in about 50% methanol.

#### 20 Preparation of xyloglucan-impregnated paper

Whatman No.1 filter paper is moistened with a 1% aqueous  
solution of xyloglucan and dried. The XLLGol-SR preparation is  
diluted into enough 75% aqueous acetone to give an absorbance at  
25 580 nm ( $A_{580}$ ) of 0.2; the xyloglucan-coated sheet of Whatman No. 1  
paper is then dipped through this solution and re-dried; the  
product is referred to as "XET-paper". Suitably sized pieces  
(e.g. 72 x 108 mm) of the XET-paper may then be glued with a non-  
aqueous adhesive onto a non-absorbent medium such as a sheet of  
30 transparent  
acetate.

#### Dot-blot test for XET activity

35       (i) A spot of the solution to be tested for XET-activity is  
pipetted on to a marked position in a piece of XET-paper. If the  
spots are 4  $\mu$ l, the spacing between the samples can conveniently  
be 9 mm (centre-to-centre, i.e. as in a standard 96-well test  
plate format).

5 (ii) The XET paper is then quickly (before the spots have dried) clamped between two sheets of plastic (e.g. acetate sheets, as used on overhead projectors) and incubated e.g. at 20°C for 1 hour.

10 (iii) The incubated XET-paper and its plastic backing is then placed (paper-side down) in a dish containing about 150 ml of a solvent [e.g. freshly prepared ethanol/formic acid/water (1:1:1 by volume)] that will remove from the paper the unreacted XLLGol-SR but not any XLLGol-SR that has become incorporated into  
15 the xyloglucan owing to XET-catalysed transglycosylation. The paper now readily detaches from the plastic backing.

(iv) The paper is then rinsed in running water for 5  
20 minutes, then in approximately 100 ml of acetone for 5 minutes, and then dried thoroughly. If desired, drying can be expedited by a 5-minute treatment in an oven at 80°C.

(v) The paper is then examined under a short-wavelength  
25 ultraviolet lamp (e.g. emitting at 254 nm; suitable eye- and skin-protection should be worn). Active XET is indicated by a pink (orange-fluorescing) spot, which can be quantified, e.g. by use of a scanning spectrofluorimeter.

### 30 XET enzymes

We have discovered that microbial enzymes with XET activity may be either transglycosylases (meaning that they lack hydrolase activity or only have a very low hydrolase activity) or they may catalyze both transglycosylation and hydrolysis of xyloglucan.

35 XET enzymes with both transglycosylating and hydrolytic activities are also described for XET enzymes obtained from plants (see Annu. Rev. Plant Physiol. Plant Mol. Biol. 1995.46: p. 509).

5 Taxonomic classification

The taxonomic classification used herein builds primarily on the system used in the :NIH Data Base (Entrez, version spring 1996) available on World Wide Web: (<http://www3.ncbi.nlm.nih.gov/htbin/ef/entrezTAX>).

10 Regarding classification of organisms which are not included in the Entrez data base the following generally available and world wide accepted reference books have been used:

For Ascomycetes: Eriksson, O.E. & Hawksworth, D.L.: Systema Ascomycetum vol 12 (1993).

For Basidiomycetes, Jülich, W.: Higher Taxa of Basidiomycetes, Bibliotheca Mycologia 85, 485pp (1981).

For Zygomycetes: O'Donnell, K.: Zygomycetes in culture, University of Georgia, US, 257pp (1979).

20 General mycological reference books:

Hawksworth, D.L., Kirk, P.M., Sutton, B.C. and Pegler, D.N.: Dictionary of the fungi, International Mycological Institute, 616 pp (1995);

Von Arx, J. A.: The genera of fungi sporulating in culture, 424 pp (1981).

Preferred fungi

30 In a preferred embodiment of the present invention the XET preparation is produced by cultivation of a fungus, in particular a fungus which belongs to *Basidiomycota*, *Zygomycota*, *Ascomycota* or a Mitosporic fungus.

35 A preferred *Basidiomycota* strain is a *Hymenomycetes* strain belonging to the orders *Coriolales*, *Schizophyllales*, *Stereales* or *Xenasmatales*; in particular a strain belonging to one of the families *Coriolaceae*, *Corticaceae*, *Schizophyllaceae*, *Stereaceae* or *Tubulicrinaceae*. Preferred genera is one of the following: *Trametes*, *Corticium*, *Schizophyllum*, or *Tubulicrinis*. Preferred species is one of the following *Trametes hirsuta*, *Corticium roseum*, *Schizophyllum* sp, *Stereum hirsutum* or *Tubulicrinis*



5    *subulatus*.

Preferred Ascomycota are strains belonging to the classes Loculoascomycetes, Discomycetes, Pyrenomycetes, and Plectomycetes, preferably those belonging to the orders Dothideales, Rhytismatales, Pezizales, Leotiales, Xylariales, Hypocreales, Halosphaeriales, Phyllachorales, Diaporthales and Eurotiales.

Preferred strains are strains belonging to the families Botryosphaeriaceae, Dothioraceae, Mycosphaerellaceae, Tubeufiaceae, Pleosporaceae, Leptosphaeriaceae, Rhytismataceae, Sarcosomataceae, Pyronemataceae, Ascobolaceae, Sclerotiniaceae, Amphisphaeriaceae, Xylariaceae, Hypocreaceae, Halosphaeriaceae, Phyllachoraceae, Valsaceae, Melanconidaceae and Trichocomataceae; especially strains belonging to the genera *Diplodia*, *Plowrightia*, *Phyllosticta*, *Septoria*, *Tubeufia*, *Alternaria*, *Coniothyrium*, *Phoma*, *Embellisia*, *Tiarosporella*, *Galiella*, *Pseudoplectania*, *Pyronema*, *Oedocephalum*, *Botrytis*, *Aposphaeria*, *Pestalotia*, *Pestalotiopsis*, *Poronia*, *Nodulisporium*, *Xylaria*, *Fusarium*, *Verticillium*, *Volutella*, *Chaetapiospora*, *Lulworthia*, *Colletotrichum*, *Cytospora*, *Discula*, *Phomopsis*, *Coryneum*, *Seimatosporium*, *Aspergillus*, *Eurotium*, *Eupenicillium*, *Penicillium*, *Petromyces* and *Talaromyces*.

Preferred are the species *Diplodia gossypina*, *Plowrightia ribesia*, *Phyllosticta* sp, *Septoria* sp, *Tubeufia amazonensis*, *Alternaria* sp, *Embellisia hyacinthi*, *Phoma neoloba*, *Phoma tropica*, *Coniothyrium* sp, *Coniothyrium olivaceum*, *Coniothyrium dunckii*, *Tiarosporella phaseolina*, *Tiarosporella* sp, *Galiella celebica*, *Pseudoplectania nigrella*, *Pyronema domesticum*, *Oedocephalum* sp, *Botrytis cinerea*, *Aposphaeria* sp, *Pestalotia* sp, *Pestalotiopsis* sp. *Poronia punctata*, *Xylaria* sp, *Nodulisporium* sp, *Fusarium solani*, *Verticillium* sp, *Volutella buxi*, *Chaetapiospora rhododendri*, *Lulworthia uniseptata*, *Colletotrichum aculatum*, *Colletotrichum crassipes*, *Cytospora* spp,

5 *Discula* sp, *Phomopsis ilicis*, *Phomopsis cirsii*, *Coryneum*  
*castaneicola*, *Seimatosporium lichenicola*, *Aspergillus tamaraii*,  
*Eurotium chevalieri*, *Eupenicillium javanicum*, *Penicillium*  
*capsulatum*, *Penicillium olsonii*, *Penicillium pinophilum*,  
10 *Penicillium roqueforti*, *Penicillium italicum*, *Penicillium*  
*canescens*, *Penicillium verruculosum*, *Petromyces alliaceus* and  
*Talaromyces flavus*.

Examples of useful Zygomycota are strains belonging to the order Mucorales, preferably strains belonging to the families Chaetocladiaceae and Mucoraceae.

15 Preferred strains belong to the genera *Dichotomocladium*, *Actinomucor*, *Gongronella*, *Sporodiniella* and *Mucor*, in particular *Dichotomocladium hesseltinei*, *Actinomucor elegans*, *Gongronellla butleri*, *Sporodiniella umbellata* and *Mucor miehei* var *minor*.

20 Example of a strain of uncertain taxonomy is *Vialaea insculpta*.

Examples of strains belonging to the Mitosporic  
25 Fungi are *Acrodontium crateriforme*, *Aureobasidium pullulans*, *Circinotrichum* sp, *Cryptocline* sp. *Ellisiopsis* sp, *Epicoccum nugrum*, *Gliocladium* sp, *Helicorhoidion irregulare*, *Hendersonia* spp, *Mariannaea* sp, *Microsphaeropsis* sp, *Ramularia* sp, *Sarcopodium* sp, *Spadicoides* sto, *Speiropsis pedatospora*,  
30 *Sporotrichum exile*, *Stilbella* sp, *Trichothecium* sp, *Trimmatostroma abietes*, *Tubakia dryina*, *Wiesneriomyces* sp and *Zygosporium masonii*.

#### Preferred bacteria

35 In another aspect, the invention relates to a novel XET preparation which is producible by cultivation of a bacterium.

Preferred bacteria are gram-negative or gram-positive.

5                Examples of XET-producing gram-positive bacteria are strains belonging to the genus *Bacillus*.

Preferred strains

The following strains have been found to be XET-positive:

10            1. *Dichotomocladium hesseltinei*. Acc No of strain: CBS 164.61.  
Classification: *Zygomycota, Mucorales, Chaetocladiaceae*.

             2. *Actinomucor elegans*. Ex of Acc No of strain: CBS 154.86.  
15 Classification: *Zygomycota, Mucorales, Chaetocladiaceae*.

             3. *Mucor miehei* var *minor*. Acc No of strain: ATCC 36018.  
Classification: *Zygomycota, Mucorales, Mucoraceae*.

             4. *Gongronella butleri*. A strain of *Gongronella butleri* has been  
deposited according to the Budapest Treaty on the International  
20 Recognition of the Deposits of Microorganisms for the Purpose of  
Patent Procedures, on 28 January 1997, at Centraalbureau voor  
Schimmelcultures (CBS), under Accession No. CBS 448.97.  
Classification: *Zygomycota, Mucorales, Chaetocladiaceae*.

             5. *Sporodiniella umbellata*. Acc No of strain: CBS 195.77.  
25 Classification: *Zygomycota, Mucorales, Mucoraceae*.

             6. *Phyllosticta* sp. Isolated from a leaf of *Pithecolobium* sp  
from China. Classification: *Ascomycota, Loculoascomycetes,*  
*Dothidiales, Mycosphaellaceae*.

             7. *Septoria* sp. A strain of *Septoria* sp has been deposited  
30 according to the Budapest Treaty on the International  
Recognition of the Deposits of Microorganisms for the Purpose of  
Patent Procedures, on 2 January 1996, at Centraalbureau voor  
Schimmelcultures (CBS), under Accession No. CBS 831.95.

- 5 Classification: *Ascomycota, Loculoascomycetes, Dothidiales, Mycosphaellaceae.*
8. *Diplodia gossypina*. A strain of *Diplodia gossypina* has been deposited according to the Budapest Treaty on the International Recognition of the Deposits of Microorganisms for the Purpose of  
10 Patent Procedures, on 12 March 1996, at Centraalbureau voor Schimmelcultures (CBS), under Accession No. CBS 274.96. Classification: *Ascomycota, Loculoascomycetes, Dothidiales, Botrysphaeriaceae.*
9. *Plowrightia ribesia*. Isolated from *Ribes* sp., Denmark.  
15 Classification: *Ascomycota, Loculoascomycetes, Dothidiales, Dothioraceae.*
10. *Tubeufia amazonensis*. Acc No of Strain: ATCC 42524. Classification: *Ascomycota, Loculoascomycetes, Dothidiales, Tubeufiaceae.*
- 20 11. *Alternaria* sp. Classification: *Ascomycota, Loculoascomycetes, Dothidiales, Pleosporaceae.*
12. *Embellisia hyacinthi*. Acc No of species IMI 211561. Classification: *Ascomycota, Loculoascomycetes, Dothidiales, Pleosporaceae.*
- 25 13. *Phoma neoloba*. Classification: *Ascomycota, Loculoascomycetes, Dothideales, Pleosporaceae.*
14. *Phoma tropica*. Ex on Acc No of species CBS 537.66. Classification: *Ascomycota, Loculoascomycetes, Dothideales, Pleosporaceae.*
- 30 15. *Coniothyrium* sp. Classification: *Ascomycota, Loculoascomycetes, Dothideales, Leptosphaeriaceae.*
16. *Coniothyrium olivaceum*. Ex on Acc No of species CBS 304.68. Classification: *Ascomycota, Loculoascomycetes, Dothideales,*

5 *Leptosphaeriaceae*.

17. *Coniothyrium dunckii*. Classification: Ascomycota, Loculoascomycetes, Dothideales, Leptosphaeriaceae.

18. *Tiarosporella phaseolina*. A strain of *Tiarosporella phaseolina* (*Macrophomina* sp) has been deposited according to the  
10 Budapest Treaty on the International Recognition of the Deposits of Microorganisms for the Purpose of Patent Procedures, on the 28<sup>th</sup> January 1997, at Centraalbureau voor Schimmelcultures (CBS) under Accession No. CBS 446.97. Classification: Ascomycota, Discomycetes, Rhytismatales, Rhytismataceae.

15 19. *Tiarosporella* sp. Classification: Ascomycota, Discomycetes, Rhytismatales, Rhytismataceae.

20. *Galiella celebica*. Isolated from a sample collected in Japan. Classification: Ascomycota, Discomycetes, Pezizales, Sarcosomataceae.

20 21. *Pseudoplectania nigrella*. A strain of *Pseudoplectania nigrella* has been deposited according to the Budapest Treaty on the International Recognition of the Deposits of Microorganisms for the Purpose of Patent Procedures, on 28 January 1997, at Centraalbureau voor Schimmelcultures (CBS), under Accession No.  
25 CBS 444.97. Classification: Ascomycota, Discomycetes, Pezizales, Sarcosomataceae.

22. *Pyronema domesticum*. Isolated from a sample from Norway. Classification: Ascomycota, Discomycetes, Pezizales, Pyronemataceae.

30 23. *Oedocephalum* sp. Classification: Ascomycota, Discomycetes, Pezizales, Ascobolaceae.

24. *Botrytis cinerea*. A strain of *Botrytis cinerea* has been deposited according to the Budapest Treaty on the International

- 5 Recognition of the Deposits of Microorganisms for the Purpose of  
Patent Procedures, on 28 January 1997, at Centraalbureau voor  
Schimmelcultures (CBS), under Accession No. CBS 447.97.  
Classification: Ascomycota, Discomycetes, Leotiales,  
Sclerotiniaceae.
- 10 25. *Aposphaeria* sp. Classification: Ascomycota, Discomycetes,  
Leotiales, Sclerotiniaceae.
26. *Pestalotia* sp. A strain of *Pestalotia* sp. has been deposited  
according to the Budapest Treaty on the International  
Recognition of the Deposits of Microorganisms for the Purpose of  
15 Patent Procedures, on 28 January 1997, at Centraalbureau voor  
Schimmelcultures (CBS), under Accession No. CBS 445.97.  
Classification: Ascomycota, Pyrenomycetes, Xylariales,  
Amphisphaeriaceae.
27. *Pestalotiopsis* sp. Classification: Ascomycota,  
20 Pyrenomycetes, Xylariales, Amphisphaeriaceae.
28. *Poronia punctata*. Isolated from a sample from Sweden.  
Classification: Ascomycota, Pyrenomycetes, Xylariales,  
Xylariaceae.
29. *Xylaria* sp. Isolated from a leaf of the palm, *Sabal*  
25 *jamaicensis*, growing in Mona, Jamaica. Classification:  
*Ascomycota, Pyrenomycetes, Xylariales, Xylariaceae Xylariaceae.*
30. *Nodulisporium* sp. Classification: Ascomycota, Pyrenomycetes,  
Xylariales, Xylariaceae.
31. *Fusarium solani*. Isolated from a sample of grain of maize  
30 from India. Classification: Ascomycota, Pyrenomycetes,  
*Hypocreales, Hypocreaceae.*
32. *Verticillium* sp. A strain of *Verticillium* sp has been  
deposited according to the Budapest Treaty on the International  
Recognition of the Deposits of Microorganisms for the Purpose of

5 Patent Procedures, on 2 January 1996, at Centraalbureau voor Schimmelcultures (CBS), under Accession No. CBS 830.95. Classification: *Ascomycota*, *Pyrenomycetes*, *Hypocreales*, *Hypocreaceae*.

33. *Volutella buxi*. Acc No of Strain: IMI 049467.  
10 Classification: *Ascomycota*, *Pyrenomycetes*, *Hypocreales*, *Hypocreaceae*.

34. *Chaetapiospora rhododendri*. Classification: *Ascomycota*, *Pyrenomycetes*, *Xylariales*, *Xylariaceae*, *Hyponectriaceae*.

35. *Lulworthia uniseptata*. A strain of *Lulworthia uniseptata* has  
15 been deposited according to the Budapest Treaty on the International Recognition of the Deposits of Microorganisms for the Purpose of Patent Procedures, on 28 January 1997, at Centraalbureau voor Schimmelcultures (CBS), under Accession No. CBS 442.97. Classification: *Ascomycota*, *Pyrenomycetes*,  
20 *Halosphaeriales*, *Halosphaeriaceae*.

36. *Colletotrichum aculatum*. Classification: *Ascomycota*, *Pyrenomycetes*, *Phyllachorales*, *Phyllachoraceae*.

37. *Colletotrichum crassipes*. Classification: *Ascomycota*, *Pyrenomycetes*, *Phyllachorales*, *Phyllachoraceae*.

25 38. *Cytospora* sp. A strain of *Cytospora* sp has been deposited according to the Budapest Treaty on the International Recognition of the Deposits of Microorganisms for the Purpose of Patent Procedures, on 23 January 1997, at Centraalbureau voor Schimmelcultures (CBS), under Accession No. CBS 424.97.  
30 Classification: *Ascomycota*, *Pyrenomycetes*, *Diaporthales*, *Valsaceae*.

39. *Cytospora* sp. A strain of *Cytospora* sp has been deposited according to the Budapest Treaty on the International Recognition of the Deposits of Microorganisms for the Purpose of  
35 Patent Procedures, on 23 January 1997, at Centraalbureau voor

- 5 Schimmelcultures (CBS), under Accession No. CBS 425.97.  
Classification: Ascomycota, Pyrenomycetes, Diaporthales,  
Valsaceae.
40. *Discula* sp. Classification: Ascomycota, Pyrenomycetes,  
Diaporthales, Valsaceae.
- 10 41. *Phomopsis ilicis*. Classification: Ascomycota, Pyrenomycetes,  
Diaporthales, Valsaceae.
42. *Phomopsis cirsii*. Classification: Ascomycota, Pyrenomycetes,  
Diaporthales, Valsaceae.
43. *Coryneum castaneicola*. Classification: Ascomycota,  
15 Pyrenomycetes, Diaporthales, Melanconidaceae.
44. *Seimatosporium lichenicola*. Classification: Ascomycota,  
Pyrenomycetes, Diaporthales, Melanconidaceae.
45. *Aspergillus tamarii*. Ex of Acc No of strain: CBS 821.72.  
Classification: Ascomycota, Plectomycetes, Eurotiales,  
20 Trichocomataceae.
46. *Eurotium chevalieri*. Ex of Acc No of strain: CBS 472.91.  
Classification: Ascomycota, Plectomycetes, Eurotiales,  
Trichocomataceae.
47. *Penicillium capsulatum*. Ex of Acc No of strain: CBS 273.86.  
25 Classification: Ascomycota, Plectomycetes, Eurotiales,  
Trichocomataceae.
48. *Penicillium olsonii*. Ex of Acc No of strain: CBS 523.89.  
Classification: Ascomycota, Plectomycetes, Eurotiales,  
Trichocomataceae.
- 30 49. *Penicillium pinophilum*. Ex of Acc No of strain: CBS 440.89.  
Classification: Ascomycota, Plectomycetes, Eurotiales,  
Trichocomataceae.



- 5 50. *Penicillium roqueforti*. Ex of Acc No of strain: CBS 167.91.  
Classification: *Ascomycota*, *Plectomycetes*, *Eurotiales*,  
*Trichocomataceae*.
51. *Penicillium italicum*. Ex of Acc No of strain: IMI 078 681.  
Classification: *Ascomycota*, *Plectomycetes*, *Eurotiales*,  
10 *Trichocomataceae*.
52. *Penicillium canescens*. Ex of Acc No of strain: CBS 579.70.  
Isolated from a salt mine in Egypt. Classification: *Ascomycota*,  
*Plectomycetes*, *Eurotiales*, *Trichocomataceae*.
53. *Eupenicillium javanicum*. Ex of Acc No of the strain: CBS  
15 448.74. Classification: *Ascomycota*, *Plectomycetes*, *Eurotiales*,  
*Trichocomataceae*.
54. *Penicillium verruculosum*. Ex of Acc No of strain: CBS  
563.92. Classification: *Ascomycota*, *Plectomycetes*, *Eurotiales*,  
*Trichocomataceae*.
- 20 55. *Talaromyces flavus*. Acc No of the strain: ATCC 52201.  
Classification: *Ascomycota*, *Plectomycetes*, *Eurotiales*,  
*Trichocomataceae*.
56. *Petromyces alliaceus*. Acc No of strain: CBS 511.69.  
Classification: *Ascomycota*, *Plectomycetes*, *Eurotiales*,  
25 *Trichocomataceae*.
57. *Trametes hirsuta*. Isolated from a sample collected in  
Denmark. Classification: *Basidiomycota*, *Hymenomycetes*,  
*Coriolales*, *Coriolaceae*.
58. *Schizophyllum* sp. A strain of *Schizophyllum* sp has been  
30 deposited according to the Budapest Treaty on the International  
Recognition of the Deposits of Microorganisms for the Purpose of  
Patent Procedures, on 28 January 1997, at Centraalbureau voor  
Schimmelcultures (CBS), under Accession No. CBS 443.97.

- 5    Classification: *Basidiomycota*, *Hymenomycetes*, *Schizophyllales*,  
*Schizophyllaceae*.
59. *Corticium roseum*. Isolated from a sample collected in  
Denmark.    Classification:    *Basidiomycota*,    *Hymenomycetes*,  
*Aleurodiscales Cortiaceae*.
- 10   60. *Tubulicrinis subulatus*. Isolated from a sample collected in  
Denmark.    Classification:    *Basidiomycota*,    *Hymenomycetes*,  
*Xenasmatales*, *Tubulicrinaceae*.
61. *Stereum hirsutum*. Isolated from a sample collected in  
Denmark.    Classification:    *Basidiomycota*,    *Hymenomycetes*,  
15   *Stereales*, *Stereaceae*.
62. *Acrodontium crateriforme*. Classification: *Mitosporic fungus*.
63. *Aureobasidium pullulans*. Classification: *Mitosporic fungus*.
64. *Circinotrichum* sp. Classification: *Mitosporic fungus*.
65. *Cryptocline* sp. Classification: *Mitosporic fungus*.
- 20   66. *Ellisiopsis* sp. Classification: *Mitosporic fungus*.
67. *Epicoccum nigrum*. Classification: *Mitosporic fungus*.
68. *Gliocladium* sp. Classification: *Mitosporic fungus*.
69.    *Helicorhoidion irregulare*. Classification: *Mitosporic*  
*fungus*.
- 25   70. *Hendersonia* sp. Classification: *Mitosporic fungus*.
71. *Mariannaea* sp. Classification: *Mitosporic fungus*.
72. *Microsphaeropsis* sp. Classification: *Mitosporic fungus*.
73. *Ramularia* sp. Classification: *Mitosporic fungus*.
74. *Sarcopodium* sp. Classification: *Mitosporic fungus*.
- 30   75. *Spadicoides* sto. Acc No of strain IMI203428. Classification:  
*Mitosporic fungus*.
76. *Speiropsis pedatospora*. Classification: *Mitosporic fungus*.
77. *Sporotrichum exile*. Acc No of strain CBS 350.47.  
Classification: *Mitosporic fungus*.
- 35   78. *Stilbella* sp. Classification: *Mitosporic fungus*.

- 5 79. *Trichothecium* sp. Classification: Mitosporic fungus.  
80. *Trimmatostroma abietes*. Classification: Mitosporic fungus.  
81. *Tubakia dryina*. Classification: Mitosporic fungus.  
82. *Wiesneriomyces* sp . Classification: Mitosporic fungus.  
83. *Zygosporium masonii*. Classification: Mitosporic fungus.
- 10 84. *Vialaea insculpta*. Classification: Uncertain.
85. *Bacillus alcalophilus*. A strain of *Bacillus alcalophilus* has been deposited according to the Budapest Treaty on the International Recognition of the Deposits of Microorganisms for the Purpose of Patent Procedures, on 12 February 1997, at  
15 Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, under Accession No. DSM 11404.

#### Production of XET

20 The XET enzyme of the invention may be produced by aerobic cultivation of the above mentioned microbial strains on a nutrient medium containing suitable carbon and nitrogen sources, such media being known in the art.

Alternatively, the XET enzyme of the invention may be produced by aerobic cultivation of a transformed host organism  
25 containing the appropriate genetic information from, e.g., one of the above mentioned strains. Accordingly, the present invention also relates to a method for the production of a xyloglucan endotransglycosylase enzyme (XET) comprising

30 (a) culturing in a suitable nutrient medium a transformed host microorganism expressing a microbial XET under conditions conducive to the production of the XET enzyme, and

(b) subsequently recovering of the XET enzyme from the nutrient medium.

Such transformants can be prepared and cultivated by  
35 methods known in the art:

#### Cloning a DNA Sequence Encoding XET

The DNA sequence encoding a XET enzyme of the

5 invention may be isolated from any microorganism producing the XET in question, using various methods well known in the art.

First, a genomic DNA and/or cDNA library should be constructed using chromosomal DNA or messenger RNA from the organism that produces the XET to be studied. Then, if the amino  
10 acid sequence of the XET is known, homologous, labelled oligonucleotide probes may be synthesized and used to identify XET-encoding clones from a genomic library or cDNA prepared from the organism in question. Alternatively, a labelled oligonucleotide probe containing sequences homologous to a known XET gene could  
15 be used as a probe to identify XET-encoding clones, using hybridization and washing conditions of lower stringency.

Yet another method for identifying XET-encoding clones would involve inserting fragments of genomic DNA or cDNA into an expression vector, such as a plasmid, transforming XET-negative  
20 host organism with the resulting DNA library, then plating the transformed cells onto agar plates and by use of the assay described above allowing clones expressing the XET to be identified.

Alternatively, the DNA sequence encoding the enzyme  
25 may be prepared synthetically by established standard methods, e.g. the phosphoamidite method described by S.L. Beaucage and M.H. Caruthers in Tetrahedron Letters 22, 1981, pp. 1859-1869 or the method described by Matthes et al. in The EMBO J. 3, 1984, pp. 801-805. In the phosphoamidite method, oligonucleotides are  
30 synthesized, e.g., in an automatic DNA synthesizer, purified, annealed, ligated and cloned in appropriate vectors.

Finally, the DNA sequence may be of mixed genomic and synthetic origin, mixed synthetic and cDNA origin or mixed genomic and cDNA origin, prepared by ligating fragments of  
35 synthetic, genomic or cDNA origin (as appropriate, the fragments corresponding to various parts of the entire DNA sequence), in accordance with standard techniques. The DNA sequence may also be prepared by polymerase chain reaction (PCR) using specific primers, for instance as described in US 4,683,202 or R.K. Saiki  
40 et al. in Science 239, 1988, pp. 487-491.

5

Expression of XET

According to the invention, a XET-encoding DNA sequence produced by methods described above, or by any alternative methods known in the art, can be expressed, in enzyme form, using an expression vector which typically includes control sequences encoding a promoter, operator, ribosome binding site, translation initiation signal, and, optionally, a repressor gene or various activator genes.

The recombinant expression vector carrying the DNA sequence encoding a XET enzyme of the invention may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g., a plasmid, a bacteriophage or an extrachromosomal element, minichromosome or an artificial chromosome. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA sequence encoding a XET of the invention, especially in a bacterial host, are the promoter of the *lac* operon of *E.coli*, the *Streptomyces coelicolor* agarase gene *dagA* promoters, the promoters of the *Bacillus licheniformis*  $\alpha$ -amylase gene (*amyL*), the promoters of the *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), the promoters of the *Bacillus Amyloliquefaciens*  $\alpha$ -amylase (*amyQ*), the promoters of the *Bacillus subtilis* *xylA* and *xylB* genes etc. For transcription in a fungal host,

5 examples of useful promoters are those derived from the gene encoding *A. oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *A. niger* neutral  $\alpha$ -amylase, *A. niger* acid stable  $\alpha$ -amylase, *A. niger* glucoamylase, *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase or  
10 *A. nidulans* acetamidase.

The expression vector of the invention may also comprise a suitable transcription terminator and, in eukaryotes, polyadenylation sequences operably connected to the DNA sequence encoding the XET enzyme of the invention. Termination and poly-  
15 adenylation sequences may suitably be derived from the same sources as the promoter.

The vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. Examples of such sequences are the origins of replication of  
20 plasmids pUC19, pACYC177, pUB110, pE194, pAMB1 and pIJ702.

The vector may also comprise a selectable marker, e.g., a gene the product of which complements a defect in the host cell, such as the *dal* genes from *B. subtilis* or *B. li-*  
*cheniformis*, or one which confers antibiotic resistance such as  
25 ampicillin, kanamycin, chloramphenicol or tetracyclin resistance. Furthermore, the vector may comprise *Aspergillus* selection markers such as *amdS*, *argB*, *niaD* and *sC*, a marker giving rise to hygromycin resistance, or the selection may be accomplished by co-transformation, e.g., as described in WO 91/17243.

30 While intracellular expression may be advantageous in some respects, e.g., when using certain bacteria as host cells, it is generally preferred that the expression is extracellular.

Procedures suitable for constructing vectors of the invention encoding a XET enzyme and containing the promoter, terminator and other elements, respectively, are well known to  
35 persons skilled in the art (cf., for instance, Sambrook et al. in Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor, 1989).

The cell of the invention, either comprising a DNA

5 construct or an expression vector of the invention as defined above, is advantageously used as a host cell in the recombinant production of a XET of the invention. The cell may be transformed with the DNA construct of the invention encoding the XET conveniently by integrating the DNA construct (in one or more copies) in  
10 the host chromosome. This integration is generally considered to be an advantage as the DNA sequence is more likely to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome may be performed according to conventional methods, e.g., by homologous or heterologous recombination.  
15 Alternatively, the cell may be transformed with an expression vector as described above in connection with the different types of host cells.

The cell of the invention may be a cell of a higher organism such as a mammal or an insect, but is preferably a  
20 microbial cell, e.g., a bacterial or a fungal (including yeast) cell.

Examples of suitable bacteria are grampositive bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*,  
25 *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus megaterium*, *Bacillus thuringiensis*, or *Streptomyces lividans* or *Streptomyces murinus*, or gramnegative bacteria such as *E.coli*. The transformation of the bacteria may, for instance, be effected  
30 by protoplast transformation or by using competent cells in a manner known *per se*.

The yeast organism may favourably be selected from a species of *Saccharomyces* or *Schizosaccharomyces*, e.g., *Saccharomyces cerevisiae*. The filamentous fungus may advantageously  
35 belong to a species of *Aspergillus*, e.g., *Aspergillus oryzae* or *Aspergillus niger*. Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known *per se*. A suitable procedure for transformation of

5 *Aspergillus* host cells is described in EP 238 023.

In a yet further aspect, the present invention relates to a method of producing a XET enzyme of the invention, which method comprises cultivating a host cell as described above under conditions conducive to the production of the XET and recovering  
10 the XET from the cells and/or culture medium.

The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in question and obtaining expression of the XET of the invention. Suitable media are available from commercial suppliers or may be  
15 prepared according to published recipes (e.g., as described in catalogues of the American Type Culture Collection).

The XET secreted from the host cells may conveniently be recovered from the culture medium by well-known procedures, including separating the cells from the medium by centrifugation  
20 or filtration, and precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by the use of chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

## 25 Industrial Applications

According to the present invention a cellulosic material may get improved strength properties and/or improved shape-retention properties and/or improved anti-wrinkling properties after treatment with a XET enzyme. The XET enzyme  
30 has the ability to rearrange and link the xyloglucan molecules which are hydrogen bonded to the cellulosic fibres whereby the above mentioned features may be achieved.

In order to enhance the effect of the XET enzyme it may in some cases be an advantage to add xyloglucan to the cellulosic  
35 material whereby the enzyme may be able to link more cellulosic material together.

The treatment of the cellulosic material with the XET enzyme may be carried out in water, or it may be carried out in water in the presence of certain components such as a buffer  
40 and/or a wetting agent and/or a stabilizer and/or a polymer



5 and/or an organic component reducing the water activity such as DMSO.

The buffer may suitably be a phosphate, borate, citrate, acetate, adipate, triethanolamine, monoethanolamine, diethanolamine, carbonate (especially alkali metal or alkaline  
10 earth metal, in particular sodium or potassium carbonate, or ammonium and HCl salts), diamine, especially diaminoethane, imidazole, Tris or amino acid buffer.

The wetting agent serves to improve the wettability of the cellulosic material. The wetting agent is preferably of a  
15 non-ionic surfactant type.

The stabilizer may be an agent stabilizing the XET enzyme.

According to the invention the concentration of XET in the aqueous medium may be 0.01-1000 µg of enzyme protein per g  
20 cellulosic material, preferably 0.05-100 µg of enzyme protein per g cellulosic material.

It will generally be appropriate to incubate the reaction medium (containing the cellulosic material and the XET enzyme) for a period of at least a few minutes. An incubation  
25 time of from 1 minute to 20 hours will generally be suitable, in particular an incubation time of from 30 minutes to 10 hours will often be preferred.

The temperature of the reaction medium in the process of the invention may suitably be in the range of 10-90°C, in  
30 particular in the range of 15-70°C, as appropriate for the XET enzyme in question.

The invention is further illustrated in the following non-limiting examples.

#### 35 **EXAMPLE 1**

##### Screening for positive XET strains.

##### 40 **Media**

5 PD Agar: 39 g potato dextrose agar, DIFCO 0013; add deionized water up to 1000 ml, autoclave at 121°C for 15 - 20 min.

YPG agar: 4 g yeast extract (DIFCO 0127),

1 g  $\text{KH}_2\text{PO}_4$  (Merck 4873),

10 0.5 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (Merck 5886)

15 g dextrose (Roquette 101-0441)

20 g agar (Merck 1614)

deionized water up to 1000 ml

Autoclave at 121° C for 15 - 20 min.

15 MEA: 20 g malt extract powder (Difco 0186)

1 g peptone (Difco 0118)

20 g glucose (Roquette France 1010441)

20 g agar (Merck 1614)

deionized water up to 1000 ml

20 Autoclave at 121°C for 15 min

Medium A. Per flask: 30 g wheat bran , 45 ml of the following solution: 10 g rofec (Roquette 101-0441), 10 g  $\text{NH}_4\text{NO}_3$  (Merck 1187), 10 g  $\text{KH}_2\text{PO}_4$  (Merck 4873), 40 g Solcafloc (Dicacel available from Dicalite-Europe-Nord, 9000 Gent, Belgium), 0.75g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (Merck 5886), 15 g  $\text{CaCO}_3$ , tap water to 1000 ml, pH adjusted to 6.5.

Autoclave for 40 min at 121° C.

- 5 Medium B. 20 g soyabean meal, 5 g maltodex 01, (Roquette 101-7845), 15 g wheat bran, 3 g peptone (Difco 0118), 10 g cellulose avicel (Merck 2331), 0.2 ml pluronic (PE-6100, 101-3068), 1 g olive oil, deionized water up to 1000 ml.
- 10 100 ml in 500 ml Erlenmeyer flask with 2 baffles. Autoclave at 121° C for 40 min.

- Medium C. 100 g sucrose, 40 g soybean meal, 10 g  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$  (Merck 6579), 0.1 ml pluronic (PE 6100), tap water up to 1000 ml. 0.5 g  $\text{CaCO}_3$  in a 2 baffled
- 15 Erlenmeyer flask with 100 ml medium. Autoclave at 121°C for 40 minutes. Just prior to use, add 10 ml of 1M  $\text{NaHCO}_3$  pH 9 per 100 ml medium.

#### FERMENTATION PROCEDURE

- 20 The fungal strains were maintained on agar in petri dishes (9 cm) or on slants with PDA, YPG or MEA (see list of media). 1 agar slant was washed off with 10 ml sterile distilled water and 3 ml were used to inoculate 1 shake flask.

- The fungal strains were grown in shake flasks under the
- 25 following growth conditions:

media: A and B (see list of media)

Temperature: 26°C

RPM: A : stationary

B : 125 - 200

- 30 Incubation time : A : 6 to 30 days

B: 2 - 21 days.

- 5 The bacterial isolate was maintained on agar slopes with TY agar pH 9 which consists of:

20 g tryptone, 5 g yeast extract, 0.7 ml of a 1% solution of  $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ , 0.1 ml of a 1% solution of  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , 1.5 ml of a 1% solution of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 20 g agar and deionized water up  
10 to 1000ml. Autoclave at 121°C for 20 minutes. Before pouring the plates, add 10 ml of sterile 1M  $\text{NaHCO}_3$  pH9 to 100 ml agar.

The slants were incubated at 30°C for 4 days. 1 slant was washed off with 10 ml sterile distilled water and 3 ml were used to inoculate each flask.

- 15 The bacterium was grown in shake flasks containing Medium C at 30°C for 4 days at 300 rpm.

#### ACTIVITY TEST:

The culture broths which were produced in B and C, were  
20 centrifuged at 10,000 rpm for 10 minutes. The supernatants were tested for XET activity.

To each flask with wheat bran and fully grown culture, was added 200 ml tap water and shaken at 200 rpm for 2 hours. The culture broth was then centrifuged and the supernatant tested for XET.

#### 25 XET ANALYSIS

The samples were analysed using the "dot-blot" assay described above.

#### RESULTS

Using the method described above, 1 *Bacillus* sp and several  
30 fungi belonging to different taxonomical groups were found to be positive. The results are shown in the table below.

The *Bacillus alcalophilus* was positive when grown on Medium C pH

5 9 (see list of media) for 4 days at 30°C at 300 rpm.

Fungal Isolate	Medium A	Medium B
<i>Actinomucor elegans</i>	+	-
<i>Dichotomocladium hesseltinei</i>	+	+
<i>Gongronella butleri</i>	not done	+
<i>Mucor miehei</i> var <i>minor</i>	+	+
<i>Sporodiniella umbellata</i>	-	+
<i>Alternaria</i> sp	+	-
<i>Aposphaeria</i>	not done	+
<i>Aspergillus tamaris</i>	+	+
<i>Botrytis cinerea</i>	+	+
<i>Chaetapiozpora rhododendri</i>	-	+
<i>Colletotrichum acutatum</i>	+	-
<i>Colletotrichum crassipes</i>	+	-
<i>Coniothyrium dunkii</i>	+	+
<i>Coniothyrium olivaceum</i>	+	-
<i>Coniothyrium</i> sp	-	+
<i>Coryneum castaneicola</i>	-	+
<i>Cytospora</i> spp	+	+
<i>Diplodia gossypina</i>	+	+
<i>Discula</i> sp	+	+
<i>Embellisia hyacinthi</i>	+	-
<i>Eupenicillium javanicum</i>	+	-

<i>Eurotium chevalieri</i>	-	+
<i>Fusarium solani</i>	+	-
<i>Galiella celebica</i>	+	+
<i>Lulworthia uniseptata</i>	+	+
<i>Nodulisporium sp</i>	+	-
<i>Oedocephalum sp</i>	-	+
<i>Penicillium canescens</i>	+	-
<i>P. capsulatum</i>	+	-
<i>P. italicum</i>	not done	+
<i>P. olsonii</i>	+	-
<i>P. pinophilum</i>	+	not done
<i>P. roqueforti</i>	+	+
<i>P. verruculosum</i>	+	+
<i>Pestalotia sp</i>	not done	+
<i>Pestalotiopsis sp</i>	+	+
<i>Petromyces alliaceus</i>	+	+
<i>Phoma neoloba</i>	+	-
<i>Phoma tropica</i>	+	+
<i>Phomopsis cirsii</i>	-	+
<i>Phomopsis ilicis</i>	+	+
<i>Phyllosticta sp</i>	+	-
<i>Plowrightia ribesia</i>	+	+
<i>Poronia punctata</i>	+	+
<i>Pseudoplectania nigrella</i>	+	+

<i>Pyronema domesticum</i>	+	+
<i>Ramularia</i> sp	+	-
<i>Seimatosporium lichenicola</i>	+	-
<i>Septoria</i> sp	+	not done
<i>Talaromyces flavus</i>	+	+
<i>Tubeufia amazonensis</i>	+	-
<i>Tiarosporella phaseolina</i>	+	+
<i>Tiarosporella</i> sp	-	+
<i>Verticillium</i> sp	+	+
<i>Volutella buxi</i>	+	-
<i>Xylaria</i> sp	+	+
<i>Corticium roseum</i>	+	+
<i>Schizophyllum</i> sp	+	+
<i>Stereum hirsutum</i>	+	+
<i>Trametes hirsuta</i>	+	+
<i>Tubulicrinis subulatus</i>	+	+
<i>Acrodontium crateriforme</i>	+	-
<i>Aureobasidium pullulans</i>	+	-
<i>Circinotrichum</i> sp	-	+
<i>Cryptocline</i> sp	+	-
<i>Ellisiopsis</i> sp	+	-
<i>Epicoccum nigrum</i>	-	+
<i>Gliocladium</i> sp	+	-
<i>Helicorhoidion irregulare</i>	+	+

<i>Hendersonia spp</i>	+	+
<i>Mariannaea sp</i>	+	-
<i>Microsphaeropsis sp</i>	+	-
<i>Ramularia sp</i>	+	-
<i>Sarcopodium sp</i>	+	-
<i>Spadicoides sp</i>	-	+
<i>Speiropsis pedatospora</i>	+	-
<i>Sporotrichum exile</i>	+	+
<i>Stilbella sp</i>	+	-
<i>Trichothecium sp</i>	+	+
<i>Trimmatostroma abietes</i>	+	+
<i>Tubakia dryina</i>	+	+
<i>Wiesneriomyces sp</i>	+	-
<i>Zygosporium masonii</i>	+	+
<i>Vialaea</i>	+	not done

5 + = positive, - = negative.

## EXAMPLE 2

### Purification and characterization of *Dichotomocladium*

10 *hesseltinei* XET.

15 PDA agar slopes were inoculated with *Dichotomocladium hesseltinei* (CBS 164.61) and incubated at 26°C for 7 days. They were washed off with about 250 ml sterile distilled water with 0.1% Tween 80 and used to inoculate 80 shake flasks containing Medium B (2 - 3ml / flask). The flasks were shaken at 200 rpm,



- 5 26°C for 5 days after which time the culture broth was centrifuged at 4000 rpm for 15 minutes.

The supernatant containing xyloglucan endotransglucosylase (XET) was purified using the following method:

- 10 Filter aid was added to the culture broth which was filtered through a filtration cloth. This solution was further filtered through a Seitz depth filter plate resulting in a clear solution. The pH of the filtrate was adjusted to pH 8.0 and the  
15 filtrate was diluted with deionised water to give the same conductivity as 20mM Tris/HCl, pH 8.0.

- The XET enzyme was applied to a Q-sepharose FF column equilibrated in 20mM Tris/HCl, pH 8.0 and the enzyme was eluted  
20 with an increasing linear NaCl gradient (0 → 0.5M). The XET activity eluted as a single peak. The pooled XET was transferred to 20mM Tris/HCl, pH 8.0 on a Sephadex G25 column and rechromatographed on a Q-sepharose FF column equilibrated in 20mM Tris/HCl, pH 8.0. The column was eluted with an increasing  
25 linear NaCl gradient (0 → 0.2M). XET containing fractions were pooled and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was added to 1.4M final concentration. A Phenyl Toyopearl 650S column was equilibrated in 1.4M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 10mM succinic acid, pH 7.0 and the XET enzyme was applied to this column and eluted with a decreasing linear (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>  
30 gradient (1.4 → 0M). XET containing fractions were pooled and concentrated on an ultrafiltration cell with a 10kDa cut-off regenerated cellulose membrane. The concentrated enzyme was applied to a Superdex200 size exclusion column equilibrated in 100mM H<sub>3</sub>BO<sub>3</sub>, 10mM dimethyl glutaric acid, 2mM CaCl<sub>2</sub>, pH 7.0.  
35 Fractions eluted from the Superdex200 column were analyzed by SDS-PAGE and pure XET fractions were pooled.

- The *Dichotomocladium hesseltinei* XET migrates on SDS-PAGE as a band with M<sub>r</sub> = 24 kDa. N-terminal amino acid sequencing of the  
40 24 kDa component was carried out following SDS-PAGE and

5 electroblotting onto a PVDF-membrane. The following N-terminal amino acid sequence (SEQ ID No. 1) could be deduced:

Ala-Glu-Phe-Cys-Gly-Gln-Trp-Asp-Thr-Gln-Thr-Val-Gly-Asn-Tyr-Ile-  
Val-Tyr-Asn-Asn-Leu-Leu-Gly-Ala-Gly-Ser-Ala.

10

The present invention also relates to a microbial XET enzyme comprising the amino acid sequence shown in SEQ ID No. 1 or a XET being at least 80% homologous with the amino acid sequence SEQ ID No.1, preferably being at least 85% homologous with SEQ ID No. 1, more preferably being at least 90% homologous with SEQ ID No.1, even more preferably being at least 95% homologous with SEQ ID No. 1, in particular being at least 98% homologous with SEQ ID No. 1.

15 A polypeptide is considered to be X% homologous to the parent XET if a comparison of the respective amino acid sequences, performed via known algorithms, such as the one described by Lipman and Pearson in Science 227, 1985, p. 1435, reveals an identity of X%.

20 In addition, mass spec. analysis of the *Dichotomocladium hesseltinei* XET gave a value of 23 006 Da.

### EXAMPLE 3

30 Purification and characterization of *Tiarosporella phaseolina* XET.

15 PDA agar slopes were inoculated with *Tiarosporella phaseolina* (CBS 446.97) and incubated at 26°C for 7 days. They were washed off with about 250 ml sterile distilled water with 0.1% Tween 80 and used to inoculate 80 shake flasks containing Medium B (2 - 3ml/flask). The flasks were shaken at 200 rpm, 26°C for 7 days after which time the culture broth was centrifuged at 4000 rpm for 15 minutes.

40

The supernatant containing xyloglucan endotransglucosylase (XET)

5 was purified using the following method:

Filter aid was added to the culture broth which was filtered through a filtration cloth. This solution was further filtered through a Seitz depth filter plate resulting in a clear  
10 solution. The filtrate was concentrated by ultrafiltration on 3 kDa cut-off polyethersulfone membranes followed by dialfiltration with distilled water to reduce the conductivity. The pH of the concentrated enzyme was adjusted to pH 5.0. The conductivity of the concentrated enzyme was 1.7 mS/cm.

15 The XET enzyme was applied to a S-sepharose FF column equilibrated in 20mM CH<sub>3</sub>COOH/NaOH, pH 5.0 and the enzyme was eluted with an increasing linear NaCl gradient (0 → 0.5M). The XET activity eluted as a single peak. The pooled XET was  
20 transferred to 20mM CH<sub>3</sub>COOH/NaOH, pH 4.0 by dialysis. The dialysed enzyme was applied to a SOURCE 30S column equilibrated in 20mM CH<sub>3</sub>COOH/NaOH, pH 4.0. After washing the column the XET activity was eluted with an increasing linear NaCl gradient (0 → 0.5M). Fractions with XET activity were pooled and dialysed  
25 against 20mM Tris/HCl, pH 8.0. The dialysed enzyme was applied to a SOURCE 30Q column equilibrated in 20mM Tris/HCl, pH 8.0. After washing the column the XET activity was eluted with an increasing linear NaCl gradient (0 → 0.5M). Fractions with XET activity were pooled and concentrated on an ultrafiltration cell  
30 with a 10kDa cut-off regenerated cellulose membrane. The concentrated enzyme was applied to a Superdex200 size exclusion column equilibrated in 100mM H<sub>3</sub>BO<sub>3</sub>, 10mM dimethyl glutaric acid, 2mM CaCl<sub>2</sub>, pH 7.0. Fractions eluted from the Superdex200 column were analyzed by SDS-PAGE and pure XET fractions were pooled.

35 The *Tiarosporella phaseolina* XET migrates on SDS-PAGE as a band with  $M_r = 24$  kDa. N-terminal amino acid sequencing of the 24 kDa component was carried out following SDS-PAGE and electroblotting onto a PVDF-membrane. The following N-terminal amino acid  
40 sequence (SEQ. ID No. 2) could be deduced:

5

Xaa-Asp-Phe-Cys-Gly-Gln-Trp-Asp-Asn-Val-Lys-Asn-Gly-Pro-Tyr-Thr-Leu-Tyr-Asn-Asn-Leu-Gly-Gly-Lys

10 The present invention also relates to a microbial XET enzyme comprising the amino acid sequence shown in SEQ ID No. 2 or a XET being at least 80% homologous with the amino acid sequence SEQ ID No. 2, preferably being at least 85% homologous with SEQ ID No. 2, more preferably being at least 90% homologous with SEQ ID No. 2, even more preferably being at least 95% homologous with SEQ ID  
15 No. 2, in particular being at least 98% homologous with SEQ ID No. 2.

A polypeptide is considered to be X% homologous to the parent XET if a comparison of the respective amino acid sequences, performed via known algorithms, such as the one described by Lipman and  
20 Pearson in Science 227, 1985, p. 1435, reveals an identity of X%.

#### EXAMPLE 4

25 **Purification and characterization of *Pseudoplectania nigrella* XET.**

15 PDA agar slopes were inoculated with *Pseudoplectania nigrella* (CBS 444.97) and incubated at 26°C for 7 days. They were washed off with about 250 ml sterile distilled water with 0.1% Tween 80  
30 and used to inoculated 80 shake flasks containing Medium B (2 - 3ml/flask). The flasks were shaken at 200 rpm, 26°C for 7 days after which time the culture broth was centrifuged at 4000 rpm for 15 minutes.

35 The supernatant containing xyloglucan endotransglycosylase (XET) was purified using the following method:

Filter aid was added to the culture broth which was filtered through a filtration cloth. This solution was further filtered  
40 through a Seitz depth filter plate resulting in a clear solution. The pH of the filtrate was adjusted to pH 5.0 and the

5 filtrate was diluted with deionised water to give the same conductivity as 20mM CH<sub>3</sub>COOH/NaOH, pH 5.0.

The XET enzyme was applied to a S-sepharose FF column equilibrated in 20mM CH<sub>3</sub>COOH/NaOH, pH 5.0 and the enzyme was  
10 eluted with an increasing linear NaCl gradient (0 → 0.25M). The XET activity eluted as a single peak. The pooled XET was transferred to 20mM Hepes/NaOH, pH 7.0 on a Sephadex G25 column and applied to a Q-sepharose FF column equilibrated in 20mM Hepes/NaOH, pH 7.0. The column was eluted with an increasing  
15 linear NaCl gradient (0 → 0.5M). XET containing fractions were pooled and transferred to 20mM CH<sub>3</sub>COOH/NaOH, pH 5.0 by dialysis. A SOURCE 15S column was equilibrated in 20mM CH<sub>3</sub>COOH/NaOH, pH 5.0, and the dialysed enzyme was applied. After washing the column the XET enzyme was eluted with an increasing linear NaCl  
20 gradient (0 → 0.2M). XET containing fractions were pooled and concentrated on an ultrafiltration cell with a 10kDa cut-off regenerated cellulose membrane. The concentrated enzyme was applied to a Superdex200 size exclusion column equilibrated in 20mM CH<sub>3</sub>COOH/NaOH, 100mM NaCl, pH 5.0. Fractions eluted from the  
25 Superdex200 column were analyzed by SDS-PAGE and pure XET fractions were pooled.

The *Pseudoplectania nigrella* XET migrates on SDS-PAGE as a band with  $M_r = 58$  kDa. Following SDS-PAGE and electroblotting onto a  
30 PVDF-membrane it was found that the 58 kDa component had a blocked N-terminus.

A highly purified preparation of *Pseudoplectania nigrella* XET was reduced and alkylated. A sample of the enzyme was then  
35 degraded with Lys-C. Peptides were isolated by RP-HPLC on a long Vydac C18 in a SMART system using TFA/AN and repurified in TFA/isopropanol. Selected peptides were analyzed by Edman degradation.

- 5 In Table 1 the sequences obtained from 8 peptides are shown. Two peptides were found to be homologous to glucanase- and xylanase-like enzymes but the majority did not show any or only irrelevant homology to known sequences.

10 **Table 1. Sequence of Lys-C peptides from *P.nigrella* XET**

Peptide	Run no.	Sequence	Comments on homology
XET lysyl 070198-2fr5	3777	WNDPVVK	Homology to B. subtilis lysis protein
XET lysyl 080198-4fr3	3778	(S/Y)RFNAPALIGE[WQ]	Nothing found
XET lysyl 090198-1fr6	3779	LIFE	Too small
XET lysyl 070198-1fr5	P379	EDGSYLKYAK	irrelevant homologies
XET lysyl 070198-1fr3	P380	EWGTTGKFENK	Homology to endoglucanase
XET lysyl 080198-1fr1	P381	KVTAVEAWK	Weak homology to xyn1_asptu. Endoxylanase
XET lysyl 080198-2fr1	P382	XFYQIANS(Q/I)	Nothing found
XET lysyl 080198-3fr1	P383	AALXXVMK	Nothing found

15 **EXAMPLE 5**

pH profile of *Dichotomocladium hesseltinei*, *Tiarosporella phaseolina* and *Pseudoplectania nigrella* XET and xyloglucanase

- 20 The xyloglucan endotransglycosylase of *Dichotomocladium hesseltinei*, *Tiarosporella phaseolina* and *Pseudoplectania*

5 *nigrella* were checked for their pH profile. The pure enzymes were diluted in buffers ranging from pH 3.0 to 11.0 so that in the final dilution, the protein concentration was the same i.e.  $A_{280} = 0.004$ . The samples were then assayed for XET using the XET-dot-blot-assay (described earlier). The fluorescent spots  
10 were judged visually and graded from 0 - 10. The units used in Fig. 1 are therefore arbitrary units.

It can be seen from Fig. 1 that the XET is active between pH 3 and pH 11, in particular between pH 4 and pH 9.

15 All the isolates with XET activity were also tested for xyloglucanase activity. The ratio between the 2 enzymes varies from isolate to isolate. The enzymes were diluted in the same buffers as for the XET pH profile activity until the final protein concentration was the same and assayed for xyloglucanase  
20 activity. AZCL xyloglucan was used. The xyloglucanase method used was the following:

Substrate: 0.4% AZCL-xyloglucan suspended in demineralised water.

25 Buffer: 100mM  $H_3BO_3$ , 10mM Dimethy glutaric acid, 2mM  $CaCl_2$ , pH 7.

Analysis:

- An Eppendorf thermomixer is switched on at 40° C
- 500 $\mu$ l 0.4% AZCL-xyloglucan is mixed with 500 $\mu$ l buffer and put  
30 on ice.
- 20 $\mu$ l enzyme is added and incubated in the Eppendorf thermomixer until a suitable colour is reached.
- The samples are returned to the ice bath to prevent further reaction while the samples are centrifuged at 0°C.
- 35 - 200  $\mu$ l samples are transferred to micro titre plates and the blue colour is measured at 650nm.

The results are shown in Fig. 2.

5    **EXAMPLE 6**

Cloning and expression of a xyloglucan endotransglycosylase enzyme (XET) from *Tiarosporella phaseolina* CBS No. 446.97

10    Deposited organisms:

*Tiarosporella phaseolina* CBS No. 446.97.

Other strains:

Yeast strain: The *Saccharomyces cerevisiae* strain used was W3124

15    (van den Hazel, H.B; Kielland-Brandt, M.C.; Winther, J.R. in Eur. J. Biochem., 207, 277-283, 1992; (MATa; ura 3-52; leu 2-3, 112; his 3-D200; pep 4-1137; prc1::HIS3; prb1:: LEU2; cir+).

*E. coli* strain: DH10B (Life Technologies)

Plasmids:

20    The *Aspergillus* expression vector pHD414 is a derivative of the plasmid p775 (described in EP 238 023). The construction of pHD414 is further described in WO 93/11249.

pYES 2.0 (Invitrogen)

Media:25    YPD:

10 g yeast extract, 20 g peptone, H<sub>2</sub>O to 900 ml. Autoclaved, 100 ml 20% glucose (sterile filtered) added.

YPM:

30    10 g yeast extract, 20 g peptone, H<sub>2</sub>O to 900 ml. Autoclaved, 100 ml 20% maltodextrin (sterile filtered) added.

10 x Basal salt:

75 g yeast nitrogen base, 113 g succinic acid, 68 g NaOH, H<sub>2</sub>O ad 1000 ml, sterile filtered.

SC-URA:

35    100 ml 10 x Basal salt, 28 ml 20% casamino acids without vitamins, 10 ml 1% tryptophan, H<sub>2</sub>O ad 900 ml, autoclaved, 3.6 ml 5% threonine and 100 ml 20% glucose or 20% galactose added.

SC-agar:

SC-URA, 20 g/l agar added.



5 AZCL Xyloglucan (Megazyme, Australia)

Expression cloning in yeast

Expression cloning in yeast was done as described by H. Dalboege et al. (H. Dalboege et al Mol. Gen. Genet (1994) 243:253-260.; WO 93/11249; WO 94/14953), which are hereby incorporated as  
10 reference. All individual steps of Extraction of total RNA, cDNA synthesis, Mung bean nuclease treatment, Blunt-ending with T4 DNA polymerase, and Construction of libraries was done according to the references mentioned above.

Fermentation procedure of *Tiarosporella phaseolina* CBS No.

15 446.97 for mRNA isolation:

*Tiarosporella phaseolina* CBS No. 446.97 was inoculated from a plate with outgrown mycelium into a shake flask containing 100 ml medium B (see media). The culture was incubated at 26°C and 200 rpm for 7 days. The resulting culture broth was filtered  
20 through miracloth and the mycelium was frozen down in liquid nitrogen.

mRNA was isolated from mycelium from this culture as described in (H. Dalboege et al Mol. Gen. Genet (1994) 243:253-260.; WO 93/11249; WO 94/14953).

25 Extraction of total RNA was performed with guanidinium thiocyanate followed by ultracentrifugation through a 5.7 M CsCl cushion, and isolation of poly(A)<sup>+</sup>RNA was carried out by oligo(dT)-cellulose affinity chromatography using the procedures described in WO 94/14953.

30 cDNA synthesis:

Double-stranded cDNA was synthesized from 5 mg poly(A)<sup>+</sup> RNA by the RNase H method (Gubler and Hoffman (1983) Gene 25:263-269, Sambrook et al. (1989) Molecular cloning: A laboratory manual, Cold Spring Harbor lab., Cold Spring Harbor, NY). The poly(A)<sup>+</sup>  
35 RNA (5 µg in 5 µl of DEPC-treated water) was heated at 70°C for 8 min. in a pre-siliconized, RNase-free Eppendorph tube, quenched on ice and combined in a final volume of 50 µl with reverse transcriptase buffer (50 mM Tris-Cl, pH 8.3, 75 mM KCl, 3 mM MgCl<sub>2</sub>, 10 mM DTT, Bethesda Research Laboratories)  
40 containing 1 mM of dATP, dGTP and dTTP and 0.5 mM 5-methyl-dCTP

5 (Pharmacia), 40 units human placental ribonuclease inhibitor (RNasin, Promega), 1.45  $\mu$ g of oligo(dT)<sub>18</sub>-Not I primer (Pharmacia) and 1000 units SuperScript II RNase H reverse transcriptase (Bethesda Research Laboratories). First-strand cDNA was synthesized by incubating the reaction mixture at 45°C  
10 for 1 hour. After synthesis, the mRNA:cDNA hybrid mixture was gelfiltrated through a MicroSpin S-400 HR (Pharmacia) spin column according to the manufacturer's instructions. After the gelfiltration, the hybrids were diluted in 250  $\mu$ l second strand buffer (20 mM Tris-Cl, pH 7.4, 90 mM KCl, 4.6 mM  
15 MgCl<sub>2</sub>, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.16 mM bNAD<sup>+</sup>) containing 200  $\mu$ l of each dNTP, 60 units *E. coli* DNA polymerase I (Pharmacia), 5.25 units RNase H (Promega) and 15 units *E. coli* DNA ligase (Boehringer Mannheim). Second strand cDNA synthesis was performed by incubating the reaction tube at 16°C for 2 hours and additional  
20 15 min. at 25°C. The reaction was stopped by addition of EDTA to a final concentration of 20 mM followed by phenol and chloroform extractions.

Mung bean nuclease treatment:

The double-stranded cDNA was precipitated at -20°C for 12 hours  
25 by addition of 2 vols 96% EtOH, 0.2 vol 10 M NH<sub>4</sub>Ac, recovered by centrifugation, washed in 70% EtOH, dried and resuspended in 30  $\mu$ l Mung bean nuclease buffer (30 mM NaAc, pH 4.6, 300 mM NaCl, 1 mM ZnSO<sub>4</sub>, 0.35 mM DTT, 2% glycerol) containing 25 units Mung bean nuclease (Pharmacia). The single-stranded hair-pin DNA was  
30 clipped by incubating the reaction at 30°C for 30 min., followed by addition of 70  $\mu$ l 10 mM Tris-Cl, pH 7.5, 1 mM EDTA, phenol extraction and precipitation with 2 vols of 96% EtOH and 0.1 vol 3 M NaAc, pH 5.2 on ice for 30 min.

Blunt-ending with T4 DNA polymerase:

35 The double-stranded cDNAs were recovered by centrifugation and blunt-ended in 30  $\mu$ l T4 DNA polymerase buffer (20 mM Tris-acetate, pH 7.9, 10 mM MgAc, 50 mM KAc, 1 mM DTT) containing 0.5 mM of each dNTP and 5 units T4 DNA polymerase (New England Biolabs) by incubating the reaction mixture at 16°C for 1 hour.  
40 The reaction was stopped by addition of EDTA to a final

5 concentration of 20 mM, followed by phenol and chloroform extractions, and precipitation for 12 hours at -20°C by adding 2 vols 96% EtOH and 0.1 vol 3 M NaAc pH 5.2.

Adaptor ligation, Not I digestion and size selection:

After the fill-in reaction the cDNAs were recovered by  
10 centrifugation, washed in 70% EtOH and dried. The cDNA pellet was resuspended in 25 µl ligation buffer (30 mM Tris-Cl, pH 7.8, 10 mM MgCl<sub>2</sub>, 10 mM DTT, 0.5 mM ATP) containing 2.5 µg non-palindromic BstXI adaptors (Invitrogen) and 30 units T4 ligase (Promega) and incubated at 16°C for 12 hours. The reaction was  
15 stopped by heating at 65°C for 20 min. and then cooling on ice for 5 min. The adapted cDNA was digested with Not I restriction enzyme by addition of 20 µl water, 5 µl 10x Not I restriction enzyme buffer (New England Biolabs) and 50 units Not I (New England Biolabs), followed by incubation for 2.5 hours at 37°C.  
20 The reaction was stopped by heating at 65°C for 10 min. The cDNAs were size-fractionated by gel electrophoresis on a 0.8% SeaPlaque GTG low melting temperature agarose gel (FMC) in 1x TBE to separate unligated adaptors and small cDNAs. The cDNAs were size-selected with a cut-off at 0.7 kb and rescued from the  
25 gel by use of b-Agarase (New England Biolabs) according to the manufacturer's instructions and precipitated for 12 hours at -20°C by adding 2 vols 96% EtOH and 0.1 vol 3 M NaAc pH 5.2.

Construction of library:

The directional, size-selected cDNAs were recovered by  
30 centrifugation, washed in 70% EtOH, dried and resuspended in 30 µl 10 mM Tris-Cl, pH 7.5, 1 mM EDTA. The cDNAs were desalted by gelfiltration through a MicroSpin S-300 HR (Pharmacia) spin column according to the manufacturer's instructions. Three test ligations were carried out in 10 µl ligation buffer (30 mM Tris-Cl, pH 7.8, 10 mM MgCl<sub>2</sub>, 10 mM DTT, 0.5 mM ATP) containing 5 µl  
35 double-stranded cDNA (reaction tubes #1 and #2), 15 units T4 ligase (Promega) and 30 ng (tube #1), 40 ng (tube #2) and 40 ng (tube #3, the vector background control) of BstXI-NotI cleaved pYES 2.0 vector. The ligation reactions were performed by  
40 incubation at 16°C for 12 hours, heating at 70°C for 20 min. and

5 addition of 10  $\mu$ l water to each tube. 1  $\mu$ l of each ligation mixture was electroporated into 40  $\mu$ l electrocompetent *E. coli* DH10B cells (Bethesda research Laboratories) as described (Sambrook et al. (1989) Molecular cloning: A laboratory manual, Cold Spring Harbor lab., Cold Spring Harbor, NY). Using the  
10 optimal conditions a library was established in *E. coli* consisting of pools. Each pool was made by spreading transformed *E. coli* on LB+ampicillin agar plates giving 15.000-30.000 colonies/plate after incubation at 37°C for 24 hours. 20 ml LB+ampicillin was added to the plate and the cells were  
15 suspended herein. The cell suspension was shaken in a 50 ml tube for 1 hour at 37°C. Plasmid DNA was isolated from the cells according to the manufacturer's instructions using QIAGEN plasmid kit and stored at -20°C.

1  $\mu$ l aliquots of purified plasmid DNA (100 ng/ml) from  
20 individual pools were transformed into *S. cerevisiae* W3124 by electroporation (Becker and Guarante (1991) Methods Enzymol. 194:182-187) and the transformants were plated on SC agar containing 2% glucose and incubated at 30°C.

#### Identification of positive colonies:

25 Colonies were screened indirectly for XET by finding xyloglucanase positive colonies.

After 3-5 days of growth, the agar plates were replica plated onto a set of SC-URA agar (with 20% galactose added) plates containing 0.1% AZCL Xyloglucan. These plates were incubated for  
30 3-7 days at 30°C. Xyloglucanase positive colonies were identified as colonies surrounded by a blue halo.

Cells from enzyme-positive colonies were spread for single colony isolation on agar, and an enzyme-producing single colony was selected for each of the Xyloglucanase-producing colonies identified.  
35

All xyloglucanase positive colonies were tested for XET and were found to be positive.

#### Isolation of a cDNA gene for expression in *Aspergillus*:

An XET-producing yeast colony was inoculated into 20 ml YPD  
40 broth in a 50 ml glass test tube. The tube was shaken for 2 days

5 at 30°C. The cells were harvested by centrifugation for 10 min. at 3000 rpm.

DNA was isolated according to WO 94/14953 and dissolved in 50 ml water. The DNA was transformed into *E. coli* by standard procedures. Plasmid DNA was isolated from *E. coli* using standard  
10 procedures, and analyzed by restriction enzyme analysis. The cDNA insert was excised using the restriction enzymes BamH I and Xba I and ligated into the *Aspergillus* expression vector pHD414 resulting in the plasmid pA2XG80.

The cDNA inset of Qiagen purified plasmid DNA of pA2XG80  
15 (Qiagen, USA) was sequenced with the Taq deoxy terminal cycle sequencing kit (Perkin Elmer, USA) and synthetic oligonucleotide primers using an Applied Biosystems ABI PRISM™ 377 DNA Sequencer according to the manufacturers instructions.

Transformation of *Aspergillus oryzae* or *Aspergillus niger*

20 Protoplasts are prepared as described in WO 95/02043, p. 16, line 21 - page 17, line 12, which is hereby incorporated by reference.

100 µl of protoplast suspension is mixed with 5-25 µg of the appropriate DNA in 10 µl of STC (1.2 M sorbitol, 10 mM Tris-HCl, pH = 7.5, 10 mM CaCl<sub>2</sub>). Protoplasts are mixed with p3SR2 (an *A. nidulans* amdS gene carrying plasmid) (Tove Christensen et al. Bio/Technology, pp 1419-1422 vol.6, Dec. 1988). The mixture is left at room temperature for 25 minutes. 0.2 ml of 60% PEG 4000 (BDH 29576), 10 mM CaCl<sub>2</sub> and 10 mM Tris-HCl, pH 7.5 is added and  
25 carefully mixed (twice) and finally 0.85 ml of the same solution is added and carefully mixed. The mixture is left at room temperature for 25 minutes, spun at 2500 g for 15 minutes and the pellet is resuspended in 2 ml of 1.2 M sorbitol. After one more sedimentation the protoplasts are spread on minimal plates  
30 (Cove, Biochem. Biophys. Acta 113 (1966) 51-56) containing 1.0 M sucrose, pH 7.0, 10 mM acetamide as nitrogen source and 20 mM CsCl to inhibit background growth. After incubation for 4-7 days at 37°C spores are picked and spread for single colonies. This  
35 procedure is repeated and spores of a single colony after the

5 second reisolation is stored as a defined transformant.

Test of *A. oryzae* transformants

Each of the *A. oryzae* transformants is inoculated in 10 ml of YPM (cf. below) and propagated. After 2-5 days of incubation at 30°C, the supernatant is removed.

10 The XET activity is identified by using the XET dot-blot assay described earlier.

The following sequence (SEQ ID No. 3) is the cDNA insert in pYES2.0 of XG80 (XET from *Tiarosporella phaseolina*) including the BamH I and Not I recognition sites used for cloning:

15 GGATCCGAATTCCAACCTATCCTGCCCTCCTTTCAAGCGAACACCATGAAGTTCTCCTCGGCTCT  
GTTTCTGGCCGCTACG  
GCGGTCTTGGCTTCCGCCGCGCCGCTTGAGCGCCGCGCCGACTTTTGTGGTCAATGGGACAACG  
TGAAGAACGGACCTTA  
20 CACTCTTTACAACAACCTGTGGGGAAAAGATGCTTCCGGAGCCTCCGGATCGCAATGCACCGGC  
GTCGATAGCTTCAGCA  
GCAACACCATCGCTTGGCACACATCCTGGTCCTGGTCCGGTGCTCAGTACAATGTCAAGTCTTA  
CGCAAACGTGGTCGTC  
GACATCACCTCTAAGAACTCAGCGCCATCAGCAGCATTAAACAGCATCTGGCGCTGGGCTTACA  
25 CGGGTAGCAACATTGT  
TGCCAATGTTGCCTACGATATCTTCACTTCATCCACTGTCTGGTGGTAGCGAGGAATATGAAATC  
ATGATATGGGTTGGTG  
CTCTCGGTGGTGCTGGTCCGATCTCATCTACCGGCTCCCCTATTGCCACCGTTTCCCTTGCAGG  
CTCCTCGTGGAAGCTC  
30 TACAAAGGGCCCAACGGGCAGATGACCGTGTTTCACTTCGTCGCCGAGTCCAACGTGAACAACT  
TCAGCGGTGACCTTAA  
CGCTTTCATCAAGTACCTCACCGGCAACCAGGGCCTTCCCGCCTCGCAATACATCAAGAGCATT  
GGCGCTGGCACTGAGC  
CGTTCACGGGTTCCAACGCCAAGCTGACCACTTCCTCCTACACTGTCAGCTTCAGATAACTGTG  
35 AAGCTTTATGCTGCCC  
TTATGCATCATCCTTGTTACATAGTTATCACCAGGGGACTCTTGTAATACGATTGCCTTATTAA  
CCGCCTGCATCTGCTT  
TCACACAATGGCATTACCAATCAACAGTGCGCCTCGAATCCGTAAAAGGTGGCTTAAAAAAA  
AAAAAAAAAAAAAAAAAA  
40 AATTCCTGCGGCCGC.

The following sequence (SEQ ID no. 4) is the amino acid sequence of the coding region of XG80:

45 MKFSSALFLAATAVLASAAPLERRADFCGQWDNVKNGPYTLNNLWGKDASGASGSQCTGVDSF  
SSNTIAWHTSWSWSGA  
QYNVKSANVVVDITSKKLSAIISSINSIWRWAYTGSNIVANVAYDIFTSSTVGGSEYEYIMIWV  
GALGGAGPISSTGSP  
ATVSLAGSSWKLYKGPNGQMTVFSFVAESNVNNSGDLNFAFIKYL TGNQGLPASQYIKSIGAGT  
50 EPFTGSNAKLTTSSYT  
VSFR.

5 The present invention also relates to a microbial XET enzyme comprising the amino acid sequence shown in SEQ ID No. 4 or a XET being at least 80% homologous with the amino acid sequence SEQ ID No. 4, preferably being at least 85% homologous with SEQ ID No. 4, more preferably being at least 90% homologous with SEQ ID No. 4, even more preferably being at least 95% homologous with SEQ ID No. 4, in particular being at least 98% homologous with SEQ ID No. 4.

10 A polypeptide is considered to be X% homologous to the parent XET if a comparison of the respective amino acid sequences, performed via known algorithms, such as the one described by Lipman and Pearson in Science 227, 1985, p. 1435, reveals an identity of X%.

A clone C1.XG80 was deposited at DSMZ on 24 February 1998 under Accession No. DSM 12032.

## 5 SEQUENCE LISTING

## (1) GENERAL INFORMATION:

## (i) APPLICANT:

(A) NAME: NOVO NORDISK A/S  
(B) STREET: Novo Alle  
(C) CITY: Bagsvaerd  
(E) COUNTRY: Denmark  
(F) POSTAL CODE (ZIP): DK-2880  
(G) TELEPHONE: +45 44 44 88 88  
(H) TELEFAX: +45 44 49 32 56

(ii) TITLE OF INVENTION: MICROBIAL XYLOGLUCAN ENDOTRANSGLYCOSYLASE  
(XET)

(iii) NUMBER OF SEQUENCES: 4

## (iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

## (2) INFORMATION FOR SEQ ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Ala Glu Phe Cys Gly Gln Trp Asp Thr Gln Thr Val Gly Asn Tyr Ile  
1 5 10 15

Val Tyr Asn Asn Leu Leu Gly Ala Gly Ser Ala  
20 25

## (2) INFORMATION FOR SEQ ID NO: 2:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Xaa Asp Phe Cys Gly Gln Trp Asp Asn Val Lys Asn Gly Pro Tyr Thr  
1 5 10 15

Leu Tyr Asn Asn Leu Gly Gly Lys



5 20

## (2) INFORMATION FOR SEQ ID NO: 3:

## (i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 975 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: DNA (genomic)

## 20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GGATCCGAAT TCCAACATATC CTGCCCTCCT TTCAAGCGAA CACCATGAAG TTCTCCTCGG 60  
CTCTGTTTCT GGCCGCTACG GCGGTCTTGG CTTCCGCCGC GCCGCTTGAG CGCCGCGCCG 120  
25 ACTTTTGTGG TCAATGGGAC AACGTGAAGA ACGGACCTTA CACTCTTTAC AACAACTGT 180  
GGGGAAAAGA TGCTTCCGGA GCCTCCGGAT CGCAATGCAC CGGCGTCGAT AGCTTCAGCA 240  
30 GCAACACCAT CGCTTGGCAC ACATCCTGGT CCTGGTCCGG TGCTCAGTAC AATGTCAAGT 300  
CTTACGCAAA CGTGGTCGTC GACATCACCT CTAAGAAACT CAGCGCCATC AGCAGCATTA 360  
ACAGCATCTG GCGCTGGGCT TACACGGGTA GCAACATTGT TGCCAATGTT GCCTACGATA 420  
35 TCTTCACTTC ATCCACTGTC GGTGGTAGCG AGGAATATGA AATCATGATA TGGGTTGGTG 480  
CTCTCGGTGG TGCTGGTCCG ATCTCATCTA CCGGCTCCCC TATTGCCACC GTTTCCCTTG 540  
40 CAGGCTCCTC GTGGAAGCTC TACAAAGGGC CCAACGGGCA GATGACCGTG TTCAGCTTCG 600  
TCGCCGAGTC CAACGTGAAC AACTTCAGCG GTGACCTTAA CGCTTTCATC AAGTACCTCA 660  
CCGGCAACCA GGGCCTTCCC GCCTCGCAAT ACATCAAGAG CATTGGCGCT GGCCTGAGC 720  
45 CGTTCACGGG TTCCAACGCC AAGCTGACCA CTTCCCTCCTA CACTGTCAGC TTCAGATAAC 780  
TGTGAAGCTT TATGCTGCCC TTATGCATCA TCCTTGTTACA TAGTTATCAC CAGGGGACTC 840  
50 TTGTAAATAC GATTGCCTTA TTAACCGCCT GCATCTGCTT TCACACAATG GCATTTACCA 900  
ATCAACAGTG CGCCTCGAAT CCGTAAAAGG TGGCTTAAAA AAAAAAAAAA AAAAAAAAAA 960  
AATTCCTGCG GCCGC 975

55

## (2) INFORMATION FOR SEQ ID NO: 4:

## (i) SEQUENCE CHARACTERISTICS:

- 60 (A) LENGTH: 244 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

65 (ii) MOLECULE TYPE: peptide

## 70 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

[illegible]

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Applicant's or agent's file reference number	5154-WO	International application No.	PCT/DK 98 / 00076
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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

10

A. The indications made below relate to the microorganism referred to in the description on page 9, line 17-22	
B. IDENTIFICATION OF DEPOSIT additional sheet	Further deposits are identified on an <input checked="checked" type="checkbox"/> additional sheet
Name of depositary institution CENTRAALBUREAU VOOR SCHIMMELCULTURES	
Address of depositary institution (including postal code and country) Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, The Netherlands	
Date of deposit 28 January 1997	Accession Number CBS 448.97
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is <input type="checkbox"/> continued on an additional sheet Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable) The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	

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Applicant's or agent's file reference number	5154-WO	International application No. PCT/DK 98 / 00076
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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

10

A. The indications made below relate to the microorganism referred to in the description on page 9, line 28 to page 10, line 5	
B. IDENTIFICATION OF DEPOSIT additional sheet	Further deposits are identified on an <input checked="" type="checkbox"/>
Name of depositary institution CENTRAALBUREAU VOOR SCHIMMELCULTURES	
Address of depositary institution (including postal code and country) Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, The Netherlands	
Date of deposit 2 January 1996	Accession Number CBS 831.95
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is <input type="checkbox"/> <del>continued on an additional sheet</del> Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	

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Applicant's or agent's file reference number	5154-WO	International application No.	PCT/DK 98 / 00076
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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page 10, lines 6-12

B. IDENTIFICATION OF DEPOSIT  
additional sheet

Further deposits are identified on an ☒

Name of depositary institution  
CENTRAALBUREAU VOOR SCHIMMELCULTURES

Address of depositary institution (including postal code and country)

Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, The Netherlands

Date of deposit  
12 March 1996

Accession Number  
CBS 274.96

C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is ☐  
continued on an additional sheet


Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

10

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page 11, lines 7-13	
B. IDENTIFICATION OF DEPOSIT additional sheet	Further deposits are identified on an <input checked="" type="checkbox"/>
Name of depositary institution CENTRAALBUREAU VOOR SCHIMMELCULTURES	
Address of depositary institution (including postal code and country) Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, The Netherlands	
Date of deposit 28 January 1997	Accession Number CBS 446.97
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/> Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable) The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	

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### INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

10

<b>A.</b> The indications made below relate to the microorganism referred to in the description on page 11, lines 20-26	
<b>B. IDENTIFICATION OF DEPOSIT</b> additional sheet	Further deposits are identified on an <span style="border: 1px solid black; padding: 2px 5px;">X</span>
Name of depositary institution CENTRAALBUREAU VOOR SCHIMMELCULTURES	
Address of depositary institution (including postal code and country) Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, The Netherlands	
Date of deposit 28 January 1997	Accession Number CBS 444.97
<b>C. ADDITIONAL INDICATIONS</b> (leave blank if not applicable) This information is <span style="border: 1px solid black; padding: 2px 5px;"></span>	
continued on an additional sheet Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.	
<b>D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE</b> (if the indications are not for all designated States)	
<b>E. SEPARATE FURNISHING OF INDICATIONS</b> (leave blank if not applicable)	
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### INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

10

<b>A.</b> The indications made below relate to the microorganism referred to in the description on page 11, line 31 to page 12, line 7	
<b>B. IDENTIFICATION OF DEPOSIT</b> additional sheet	Further deposits are identified on an <input checked="" type="checkbox"/>
Name of depositary institution CENTRAALBUREAU VOOR SCHIMMELCULTURES	
Address of depositary institution (including postal code and country) Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, The Netherlands	
Date of deposit 28 January 1997	Accession Number CBS 447.97
<b>C. ADDITIONAL INDICATIONS</b> (leave blank if not applicable) This information is <input type="checkbox"/> continued on an additional sheet	
Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.	
<b>D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE</b> (if the indications are not for all designated States)	
<b>E. SEPARATE FURNISHING OF INDICATIONS</b> (leave blank if not applicable)	
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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

10

A. The indications made below relate to the microorganism referred to in the description on page 12, lines 10-16	
B. IDENTIFICATION OF DEPOSIT additional sheet	
Further deposits are identified on an <input checked="" type="checkbox"/>	
Name of depositary institution CENTRAALBUREAU VOOR SCHIMMELCULTURES	
Address of depositary institution (including postal code and country) Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, The Netherlands	
Date of deposit 28 January 1997	Accession Number CBS 445.97
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/> Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable) The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	

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Applicant's or agent's file reference number	5154-WO	International application No.	PCT/DK 98 / 00076
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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

10

(PCT Rule 13bis)

<b>A.</b> The indications made below relate to the microorganism referred to in the description on page 12, line 30 to page 13, line 6.	
<b>B. IDENTIFICATION OF DEPOSIT</b> additional sheet	Further deposits are identified on an <input checked="" type="checkbox"/> additional sheet
Name of depositary institution CENTRAALBUREAU VOOR SCHIMMELCULTURES	
Address of depositary institution (including postal code and country) Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, The Netherlands	
Date of deposit 2 January 1996	Accession Number CBS 830.95
<b>C. ADDITIONAL INDICATIONS</b> (leave blank if not applicable) This information is <input type="checkbox"/> continued on an additional sheet. Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.	
<b>D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE</b> (if the indications are not for all designated States)  	
<b>E. SEPARATE FURNISHING OF INDICATIONS</b> (leave blank if not applicable) The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	

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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

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(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page 13, lines 12-18.	
B. IDENTIFICATION OF DEPOSIT additional sheet	Further deposits are identified on an <input checked="" type="checkbox"/> additional sheet
Name of depositary institution CENTRAALBUREAU VOOR SCHIMMELCULTURES	
Address of depositary institution (including postal code and country) Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, The Netherlands	
Date of deposit 28 January 1997	Accession Number CBS 442.97
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	

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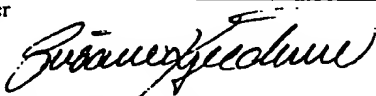
Applicant's or agent's file reference number	5154-WO	International application No.	PCT/DK 98 / 00076
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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

10

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page 13, lines 23-29.	
B. IDENTIFICATION OF DEPOSIT additional sheet	Further deposits are identified on an <input checked="" type="checkbox"/>
Name of depositary institution CENTRAALBUREAU VOOR SCHIMMELCULTURES	
Address of depositary institution (including postal code and country) Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, The Netherlands	
Date of deposit 23 January 1997	Accession Number CBS 424.97
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is <input type="checkbox"/> <del>continued on an additional sheet</del> Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	

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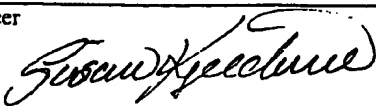
Applicant's or agent's file reference number	5154-WO	International application No.	PCT/DK 98/00076
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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

10

A. The indications made below relate to the microorganism referred to in the description on page 13, line 30 to page 14, line 6.	
B. IDENTIFICATION OF DEPOSIT additional sheet	Further deposits are identified on an <input checked="" type="checkbox"/>
Name of depositary institution CENTRAALBUREAU VOOR SCHIMMELCULTURES	
Address of depositary institution (including postal code and country) Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, The Netherlands	
Date of deposit 23 January 1997	Accession Number CBS 425.97
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/> Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	

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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page 15, line 29 to page 16, line 6.

B. IDENTIFICATION OF DEPOSIT  
additional sheet

Further deposits are identified on an ☒

Name of depositary institution  
CENTRAALBUREAU VOOR SCHIMMELCULTURES

Address of depositary institution (including postal code and country)

Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, The Netherlands

Date of deposit  
28 January 1997

Accession Number  
CBS 443.97

C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is ☐  
continued on an additional sheet

Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.

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E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

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Applicant's or agent's file  
reference number 5154-WO

International application No. PCT/DK 98/00076

## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

10

A. The indications made below relate to the microorganism referred to in the description on page 17, lines 11-16.

B. IDENTIFICATION OF DEPOSIT  
additional sheet

Further deposits are identified on an ☒

Name of depositary institution

DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH

Address of depositary institution (including postal code and country)

Mascheroder Weg 1b, D-38124 Braunschweig, GERMANY

Date of deposit

12 February 1997

Accession Number

DSM 11404

C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is ☐  
continued on an additional sheet

Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

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Applicant's or agent's file reference number 5154-WO	International application No. PCT/DK 98/00076
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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

10

A. The indications made below relate to the microorganism referred to in the description on page 45, lines 17-18.	
B. IDENTIFICATION OF DEPOSIT additional sheet	Further deposits are identified on an <input type="checkbox"/>
Name of depositary institution DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH	
Address of depositary institution (including postal code and country) Mascheroder Weg 1b, D-38124 Braunschweig, GERMANY	
Date of deposit 24 February 1998	Accession Number DSM 12032
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is <input type="checkbox"/> continued on an additional sheet	
Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
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## 5 CLAIMS

1. A method for the production of a xyloglucan endotransglycosylase enzyme (XET) comprising  
(a) culturing in a suitable nutrient medium a microorganism  
10 expressing a microbial XET under conditions conducive to the production of the XET enzyme, and  
(b) subsequently recovering of the XET enzyme from the nutrient medium.
- 15 2. The method according to claim 1, wherein the microorganism is a fungus or a bacterium.
3. The method according to claim 2, wherein the fungus is a basidiomycota, an ascomycota, a zygomycota or a mitosporic  
20 fungus.
4. The method according to claim 3, wherein the fungus is a basidiomycotinum strain of the order *Coriolales*, *Schizophyllales*, *Stereales*, or *Xenasmatales*.
- 25 5. The method according to claim 4, wherein the fungus is a basidiomycotinum selected from a strain belonging to the group consisting of the families *Coriolaceae*, *Corticaceae*, *Schizophyllaceae*, *Stereaceae* and *Tubulicrinaceae*.
- 30 6. The method according to claim 4, wherein the fungus is a basidiomycotinum selected from a strain belonging to the group consisting of the genera *Trametes*, *Corticium*, *Schizophyllum*, *Stereum* and *Tubulicrinis*.
- 35 7. The method according to claim 6, wherein the fungus is a basidiomycotinum selected from a strain belonging to the group consisting of the species *Trametes hirsuta*, *Corticium roseum*, *Schizophyllum sp*, *Stereum hirsutum* and *Tubulicrinis subulatus*.
- 40

- 5 8. The method according to claim 7, wherein the fungus is a *Schizophyllum* sp, deposit no. CBS 443.97.
9. The method according to claim 3, wherein the ascomycetes is selected from a strain belonging to the group consisting of the  
10 classes *Loculoascomycetes*, *Discomycetes*, *Pyrenomycetes*, and *Plectomycetes*.
10. The method according to claim 9, wherein the ascomycetes is selected from a strain belonging to the group consisting of the  
15 orders *Dothideales*, *Rhytismatales*, *Pezizales*, *Leotiales*, *Xylariales*, *Hypocreales*, *Halosphaeriales*, *Eurotiales*, *Phyllachorales* and *Diaporthales*.
11. The method according to claim 10, wherein the ascomycetes is  
20 selected from a strain belonging to the group consisting of the families *Leptosphaeriaceae*, *Botryosphaeriaceae*, *Dothioraceae*, *Mycosphaerellaceae*, *Tubeufiaceae*, *Rhytismataceae*, *Sarcosomataceae*, *Pyronemataceae*, *Sclerotiniaceae*, *Amphisphaeriaceae*, *Hyponectriaceae*, *Xylariaceae*, *Valsaceae*,  
25 *Melanconidaceae*, *Hypocreaceae*, *Halosphaeriaceae*, *Phyllachoraceae* and *Trichocomataceae*.
12. The method according to claim 11, wherein the ascomycetes is selected from a strain belonging to the group consisting of the  
30 genera *Coniothyrium*, *Phoma*, *Diplodia*, *Plowrightia*, *Phyllosticta*, *Septoria*, *Tubeufia*, *Alternaria*, *Embellisia*, *Tiarosporella*, *Galiella*, *Oedocephalum*, *Pseudoplectania*, *Pyronema*, *Botrytis*, *Aposphaeria*, *Pestalotia*, *Pestalotiopsis*, *Chaetapiolella*, *Poronia*, *Nodulisporium*, *Xylaria*, *Cytospora*, *Discula*, *Phomopsis*, *Coryneum*,  
35 *Seimatosporium*, *Fusarium*, *Verticillium*, *Volutella*, *Lulworthia*, *Colletotrichum*, *Aspergillus*, *Eurotium*, *Eupenicillium*, *Penicillium*, *Petromyces* and *Talaromyces*.
13. The method according to claim 12, wherein the ascomycetes is

5 selected from a strain belonging to the group consisting of the  
species *Diplodia gossypina*, *Plowrightia ribesia*, *Phyllosticta* sp,  
*Septoria* sp, *Tubeufia amazonensis*, *Alternaria* sp, *Embellisia*  
*hyacinthi*, *Phoma neoloba*, *Phoma tropica*, *Coniothyrium* sp,  
*Coniothyrium olivaceum*, *Coniothyrium dunckii*, *Tiarosporella* sp,  
10 *Tiarosporella phaseolina*, *Galiella celebica*, *Pseudoplectania*  
*nigrella*, *Pyronema domesticum*, *Oedocephalum* sp, *Botrytis cinerea*,  
*Aposphaeria* sp, *Pestalotia* sp, *Pestalotiopsis* sp, *Poronia*  
*punctata*, *Xylaria* sp, *Nodulisporium* sp, *Fusarium solani*,  
*Verticillium* sp, *Volutella buxi*, *Chaetapiolepora rhododendri*,  
15 *Lulworthia uniseptata*, *Colletotrichum acutatum*, *Colletotrichum*  
*crassipes*, *Cytospora* spp, *Discula* sp, *Phomopsis* sp, *Phomopsis*  
*cirsii*, *Coryneum castaneicola*, *Seimatosporium lichenicola*,  
*Aspergillus tamarii*, *Eurotium chevalieri*, *Eupenicillium*  
*javanicum*, *Penicillium capsulatum*, *Penicillium olsonii*,  
20 *Penicillium pinophilum*, *Penicillium roqueforti*, *Penicillium*  
*italicum*, *Penicillium verruculosum*, *Penicillium canescens*,  
*Petromyces alliaceus* and *Talaromyces flavus*.

14. The method according to claim 13, wherein the ascomycetes is  
25 selected from a strain belonging to the group consisting of the  
species *Botrytis cinerea* deposit no. CBS 447.97, *Pseudoplectania*  
*nigrella* deposit no. CBS 444.97, *Tiarosporella phaseolina* deposit  
no. CBS 446.97, *Pestalotia* sp deposit no. CBS 445.97 and  
*Lulworthia uniseptata* deposit no. CBS 442.97.

30 15. The method according to claim 3, wherein the zygomycota is  
selected from a strain belonging to the order *Mucorales*.

16. The method according to claim 15, wherein the fungus is a zy-  
35 gomycotum selected from a strain belonging to the group  
consisting of the families *Chaetocladiaceae* and *Mucoraceae*.

17. The method according to claim 16, wherein the fungus is a zy-

5 gomycotum selected from a strain belonging to the group  
consisting of the genera *Dichotomocladium*, *Actinomucor*,  
*Gongronella*, *Sporodiniella*, and *Mucor*.

10 18. The method according to claim 17, wherein the fungus is a zy-  
gomycota selected from a strain belonging to the group consisting  
of the species *Dichotomocladium hesseltinei*, *Actinomucor*  
*elegans*, *Gongronella butleri*, *Mucor miehei* var *minor* and  
*Sporodiniella umbellata*.

15 19. The method according to claim 18, wherein the fungus is a  
*Gongronella butleri* deposit no. CBS 448.97.

20 20. The method according to claim 2, wherein the fungus is a  
mitosporic fungus.

21. The method according to claim 1, wherein the microorganism is  
*Vialaea insculpta*.

25 22. The method according to claim 2, wherein the bacterium is  
gram-positive.

23. The method according to claim 22, wherein the gram-positive  
bacterium is *Bacillus*.

30 24. The method according to claim 23, wherein the gram-positive  
bacterium is *Bacillus alcalophilus* deposit no DSM 11404.

25. Use of a XET preparation obtained according to any of claims  
1-24 for treating a cellulosic material.

35 26. The use according to claim 25, wherein the treatment provides  
improved strength and/or improved shape-retention and/or improved  
anti-wrinkling properties of the cellulosic material.

5 27. The use according to claim 25, wherein the cellulosic material is a cellulosic fabric or a paper and pulp product.

28. A xyloglucan endotransglycosylase preparation which is producible by cultivation of a microorganism expressing a microbial  
10 XET.

29. The preparation according to claim 28, wherein the XET has the amino acid sequence shown in SEQ ID No. 1, or the XET has an amino acid sequence which is at least 80% homologous with SEQ ID  
15 No. 1.

30. The preparation according to claim 28, wherein the XET has the amino acid sequence shown in SEQ ID No. 2, or the XET has an amino acid sequence which is at least 80% homologous with SEQ ID  
20 No. 2.

31. The preparation according to claim 28, wherein the XET has the amino acid sequence shown in SEQ ID No. 4, or the XET has an amino acid sequence which is at least 80% homologous with SEQ ID  
25 No. 4.

32. The preparation according to claim 28, wherein the XET is active between 3 and 11, in particular between 4 and 9.

30 33. A method for the production of a xyloglucan endotransglycosylase enzyme (XET) comprising  
(a) culturing in a suitable nutrient medium a transformed host microorganism expressing a microbial XET under conditions conducive to the production of the XET enzyme, and  
35 (b) subsequently recovering of the XET enzyme from the nutrient medium.

1/2

XET units

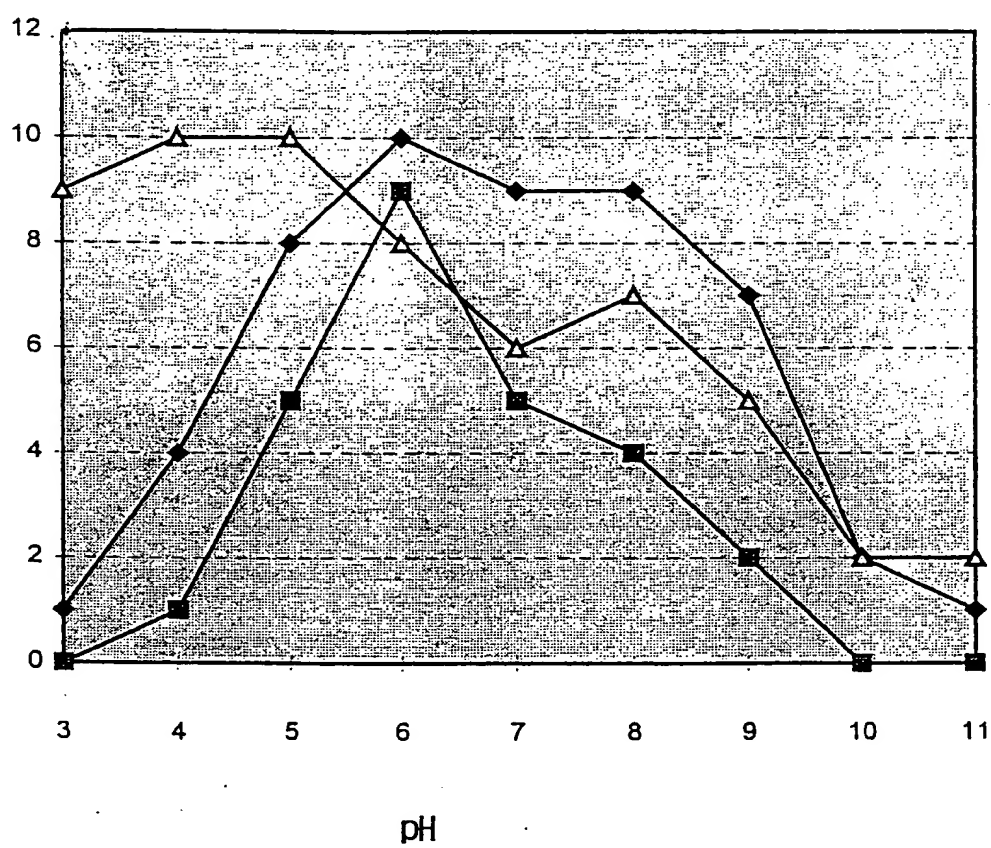


Fig. 1

2/2

XYLOGLUCANASE UNITS

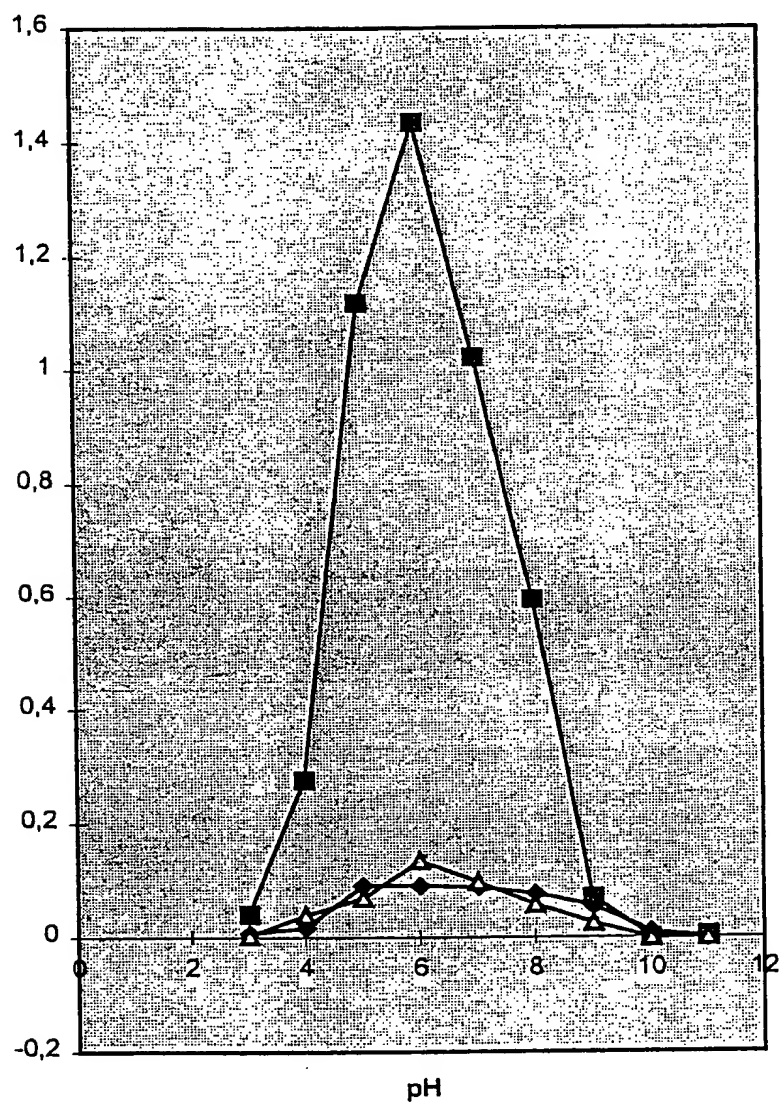


Fig. 2

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 98/00076

## A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 9/10, C12N 9/24, C12N 9/42

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CA, MEDLINE, BIOSIS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 9513384 A1 (UNILEVER PLC), 18 May 1995 (18.05.95)	1-24
X	---	25-33
A	EP 0562836 A1 (TAKARA SHUZO CO.LTD.), 29 Sept 1993 (29.09.93)	1-24
X	---	25-33

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

## \* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

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Date of the actual completion of the international search

25 May 1998

Date of mailing of the international search report

17 -06- 1998

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 98/00076

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Dialog Information Service, File Biosis, Dialog accession no. 7153746, Biosis accession no. 93138746, Fry S. C. et al: "Xyloglucan Endotransglycosylase A New Wall -Loosening Enzyme Activity From Plants", Biochem J 282 (3). 1992. 821-828	1-24
X	----- -----	25-33

**INTERNATIONAL SEARCH REPORT**  
Information on patent family members

29/04/98

International application No.  
PCT/DK 98/00076

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9513384 A1	18/05/95	AU 8112994 A	29/05/95
		CA 2176133 A	18/05/95
		CZ 9601361 A	11/12/96
		EP 0728208 A	28/08/96
		HU 74598 A	28/01/97
		HU 9601232 D	00/00/00
		JP 9511121 T	11/11/97
		PL 317046 A	03/03/97
		SK 57696 A	01/10/96
EP 0562836 A1	29/09/93	AU 667706 B	04/04/96
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		US 5516694 A	14/05/96

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